STUDY ON LOCAL PLANTS AS SOURCE OF INHIBITORS TO HUMAN GLUTATHIONE S-TRANSFERASE π (GST-P)

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FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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ABSTRACT

The detoxification function of glutathione S-transferase π (GST-P) was associated with drug resistance in many cancers and become a major reason of chemotherapy failure and disease recurrence. Thus, inhibitors of GST-P were targeted in order to counteract the phenomenon of multidrug resistance. Ethanolic extracts of 43 local plant samples were screened for their inhibitory effect on GST-P activity. Among these, 30 ethanolic extracts displayed inhibition activity on GST-P and 13 out of the 30 ethanolic extracts have inhibition effects higher than 50%. Furthermore, *Garcinia atroviridis* (branch) and *Leptospermum flavescens* (leaf) ethanolic extracts have the highest inhibitory effect with a 100% inhibition on GST-P activity. These 13 ethanolic extracts were then subjected for IC_{50} determination, kinetic studies and cytotoxicity assays. Based on the IC_{50} value, the most active sample was *Cinnamomum zeylanicum* (branch) ethanolic extract with lowest IC_{50} value of 0.07 mg/mL, followed by *Leptospermum flavescens* (leaf) and *Hibiscus tiliaceus* (leaf) ethanolic extracts with IC_{50} values of 0.09 and 0.10 mg/mL respectively. 10 of the selected ethanolic extracts shown mixed mode inhibition on GST-P while the other 3 shown uncompetitive inhibitions. All of the 13 ethanolic extracts were not cytotoxic to both HT-29 and MRC-5 cell lines when tested alone, with IC_{50} value >100 μg/mL. Combination studies indicated that GST-P inhibition able to potentiate the cytotoxicity of doxorubicin hydrochloride on HT-29 cells, but not for cisplatin. Combination of doxorubicin hydrochloride-*Cinnamomum zeylanicum* (branch) ethanolic extract has the lowest IC_{50} value with IC_{50}= 0.22 μg/mL. Nevertheless, *Andrographis paniculata* (leaf) and *Lawsonia inermis* (branch) ethanolic extracts incredibly increased the cytotoxicity of cisplatin on HT-29 cells with IC_{50} values of 4.70 and 5.46 μg/mL respectively. Bioassay-guided fractionation of *Leptospermum flavescens* (leaf) ethanolic extract on polyamide column resulted in a fraction with 95% inhibition on GST-P activity (50% methanol 2% acetic

acid eluate). The fraction inhibited GST-P in mixed mode with IC_{50} value of 0.19 mg/mL. This fraction was not toxic to either HT-29 or MRC-5 cells. Combination of doxorubicin hydrochloride with the 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract enhanced doxorubicin hydrochloride cytotoxicity on HT-29 cells with IC_{50}= 0.26 µg/mL whereas combination of cisplatin-50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract gave an IC_{50} value of 8.38 µg/mL on cytotoxicity of HT-29 cells. Our results revealed that local plants can be source of GST-P inhibitors to enhance cytotoxicity of anticancer drugs.

ABSTRAK

Fungsi detoksifikasi glutathione S-transferase π (GST-P) sering dikaitkan dengan rintangan kanser terhadap dadah dan ini menjadi punca utama kegagalan dalam rawatan kemoterapi dan pengulangan penyakit. Oleh itu, perencat kepada GST-P telah dijadikan sebagai sasaran untuk mengatasi fenomena rintangan terhadap pelbagai dadah. Kesan perencatan kepada aktiviti GST-P yang disebabkan oleh ekstrak etanolik daripada 43 sampel tumbuhan tempatan telah diuji. Di antaranya, 30 ekstrak etanolik telah menunjukkan perencatan terhadap GST-P dan 13 daripada 30 ekstrak etanolik itu mempunyai kesan perencatan lebih daripada 50%. Lagipun extrak etanolik *Garcinia atroviridis* (batang) dan *Leptospermum flavescens* (daun) mempunyai 100% perencatan terhadap aktiviti GST-P. 13 ekstrak etanolik ini telah dipilih untuk penentuan IC_{50}, kajian kinetic dan ujian-ujian kesitotoksikan. Berdasarkan kepada nilai-nilai IC_{50} yang didapati, sampel yang paling aktif adalah ekstrak etanolik *Cinnamomum zeylanicum* (batang) dengan nilai IC_{50} yang paling rendah sekali iaitu 0.07 mg/mL, diikuti dengan ekstrak etanolik daripada *Leptospermum flavescens* (daun) and *Hibiscus tiliaceus* (daun) yang mempunyai nilai IC_{50} sebanyak 0.09 and 0.10 mg/mL. 10 daripada ekstrak-ekstrak etanolik yang terpilih itu telah menunjukkan perencatan GST-P secara mod campuran manakala 3 yang lain itu menunjukkan perencatan secara tak-kompetitif. Semua ekstrak etanolik yang terpilih adalah tidak toksik kepada sel-sel HT-29 dan MRC-5 apabila diuji secara individu dengan nilai IC_{50} >100 μg/mL. Kajian-kajian gabungan menunjukkan bahawa perencatan GST-P mampu mempertingkatkan kesitotoksikan doxorubicin hidroklorida kepada sel-sel HT-29, tetapi tidak untuk cisplatin. Gabungan doxorubicin hidroklorida dengan ekstrak etanolik *Cinnamomum zeylanicum* (batang) mempunyai nilai IC_{50} yang paling rendah iaitu IC_{50}= 0.22 μg/mL. Walaubagaimanapun, ekstrak etanolik daripada *Andrographis paniculata* (daun) and *Lawsonia inermis* (batang) telah mempertingkatkan lagi kesitotoksikan cisplatin kepada sel-sel HT-29 dengan nilai IC_{50}

sebanyak 4.70 and 5.46 μg/mL. Fraksinasi berdasarkan bioassay ekstrak etanolik *Leptospermum flavescens* (daun) menggunakan kolum polyamida telah menunjukkan keputusan pecahan dengan 95% perencatan terhadap aktiviti GST-P (eluat 50% metanol 2% asid asetik). Pecahan ini telah merencatkan GST-P secara mod campuran dengan nilai IC_{50} sebanyak 0.19 mg/mL. Pecahan ini didapati tidak toksik kepada sel-sel HT-29 ataupun MRC-5. Gabungan doxorubicin hidroklorida dengan pecahan 50% metanol 2% asid asetik dari ekstrak etanolik *L. flavescens* (daun) memperkuatkan kesitotoksikan doxorubicin hidroklorida kepada sel-sel HT-29 dengan IC_{50}= 0.26 μg/mL, manakala gabungan cisplatin dengan pecahan 50% metanol 2% asid asetik dari ekstrak etanolik *L. flavescens* (daun) memberikan nilai IC_{50} sebanyak 8.38 μg/mL atas kesitotoksikan terhadap sel-sel HT-29. Keputusan kajian kami menunjukkan bahawa tunbuhan tempatan boleh dijadikan sumber perencat GST-P untuk memperkuatkan kesitotoksikan dadah antikanser.

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To them, I dedicate this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
&	And
$(NH_4)HCO_3$	Ammonium bicarbonate
˚C	Celsius (temperature in degree centigrade)
4-NQO	4-Nitroquinoline 1-oxide
A	Absorbance
ACN	Acetonitrile
Ala	Alanine
ANOVA	Analysis of variance
APS	Ammonium persulfate
Ca^{2+}	Calcium ion
CDNB	1-chloro-2,4-dinitrobenzene
CHCA	α-cyano-4-hydroxycinnamic acid
CHP	Cumene hydroperoxide
cm^2	centimeter square
CO_2	Carbon dioxide
DCM	Dichloromethane
DCNB	1,2-Dichloro-4-nitrobenzene
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
Doxo	Doxorubicin hydrochloride
EA	Ethacrynic acid
EC	Enzyme commission codes
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
et al.	and others
FPLC	Fast protein liquid chromatography
g	gram
GSH	Glutathione
GST	Glutathione S-transferase
GSTA	Glutathione S-transferase alpha
GST-M	Glutathione S-transferase mu
GST-P	Glutathione S-transferase π
h	hour
HIV	Human immunodeficiency virus
IC_{50}	Half maximal inhibitory concentration
IEF	Isoelectric focusing
Ile	Isoleucine
JNK	c-Jun N-terminal kinases

kDa	kilodalton
Ki	Inhibition constant
K_m	Michaelis constant
L-DOPA	L-3,4-dihydroxyphenylalanine
M	Molarity
mA	milliampere
MALDI-TOF	Matrix assisted laser desorption/ionization time-of-flight
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
mg	milligram
mg/L	milligram per liter
mg/mL	milligram per milliliter
mL	milliliter
mm	millimeter
mM	milliMolar
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Not determined

nm	nanometer
OD	Optical density
OvGST	*Onchocerca volvulus* GST
PBS	Phosphate buffer saline
PfGST	*Plasmodium falciparum* GST
pI	Isoelectric point
PMF	Peptide mass fingerprint
R_f	Relative mobility
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
S.D.	Standard deviation
SA	Specific activity
SDS	Sodium dodecylsulfate
TEMED	N,N,N',N'- Tetramethylethylenediamine
TER	Terrapin
TFA	Trifluoroacetic acid
TLK	Ezatiostat hydrochloride (Telintra)
UV	Ultraviolet
V	Voltage

Val	Valine
V_{max}	Maximum velocity
w/v	weight over volume
xg	times gravity
µg/mL	microgram per milliliter
µL	microliter
µM	microMolar
µmol/min	micromole per minute
µmol/min/mg	micromole per minute per milligram
π	pi

1.0 INTRODUCTION

Cytosolic Glutathione S-Transferases (GSTs; EC 2.5.1.18) constitute a family of multifunctional enzymes which catalyse the conjugation of glutathione (GSH) with a wide variety of xenobiotics (Mannervik *et al.*, 1985). Among all of the GST classes that have been characterized, Glutathione S-Transferase π (GST-P) has significance in cancer diagnosis as it was expressed ubiquitously and abundantly in maglinant cells. Elevated GST-P expression is associated with multidrug resistance which contributes to chemoresistance in many cancers and become a major reason of chemotherapy failure and disease recurrence (Huang *et al.*, 2007). In order to counteract the phenomenon of multidrug resistance which partly caused by the GST-P, specific inhibitors for this enzyme has been found and designed to sensitize those tumor cells to anticancer drugs. Since HT-29 cell line was reported to have high expression of GST-P, this cell line was selected for cytotoxicity assays and extraction of GST-P enzyme. Studies on natural products reported significant biological effects against different types of cancer. Plant polypeptide which has been extensively studied in recent decades has been targeted in this study as a potential inhibitor of GST-P.

2.0 LITERATURE REVIEW

2.1 GLUTATHIONE S-TRANSFERASES

The discovery of Glutathione S-Transferases (GST) is back to the year of 1960, where Booth *et al*. (1960; 1961) shown that there was an enzyme exist in rat liver which catalyzed the conjugation of glutathione with epoxides. The enzyme catalyzed the reaction was then partially purified and characterized as glutathione S-aryltransferase (which currently known as Glutathione S-transferase) by Grover and Sims (1964). GSTs (EC 2.5.1.18) constitute a family of multifunctional and multigene enzymes which catalyzes the conjugation of glutathione (GSH) with a wide variety of xenobiotics and endogenous compounds. The conjugations of glutathione with xenobiotics like carcinogens and exogenous drugs often produce more water soluble conjugates that usually less toxic and readily to be excreted (Mannervik *et al.*, 1985; Zhang and Fang, 1999). GSTs are involves in the metabolism of xenobiotics in the phase II detoxification process (illustrated in Figure 2.1). Besides of detoxification, the enzymes also involve in other intracellular processes such as the removal of reactive oxygen species and thiol protein regeneration (during oxidative stress). Selenium-independent glutathione peroxidase (GPx) activity of GSTs towards organic hydroperoxides prevents engagement of organic hydroperoxides of fatty acids, phospholipids and DNA in free radical propagation reactions eventually leads to destruction of macromolecules (Hayes and Strange, 1995). Furthermore, GSTs also involve in the production of leukotriene and prostaglandin, catalysis of the binding of endogenous ligands, catalysis of reactions in non-detoxification metabolic pathways and bind non-catalytically to exogenous and endogenous compounds (Dragani *et al.*, 1998; Sheehan *et al*., 2001; Kitteringham *et al*., 2007; Tew *et al*., 1988).

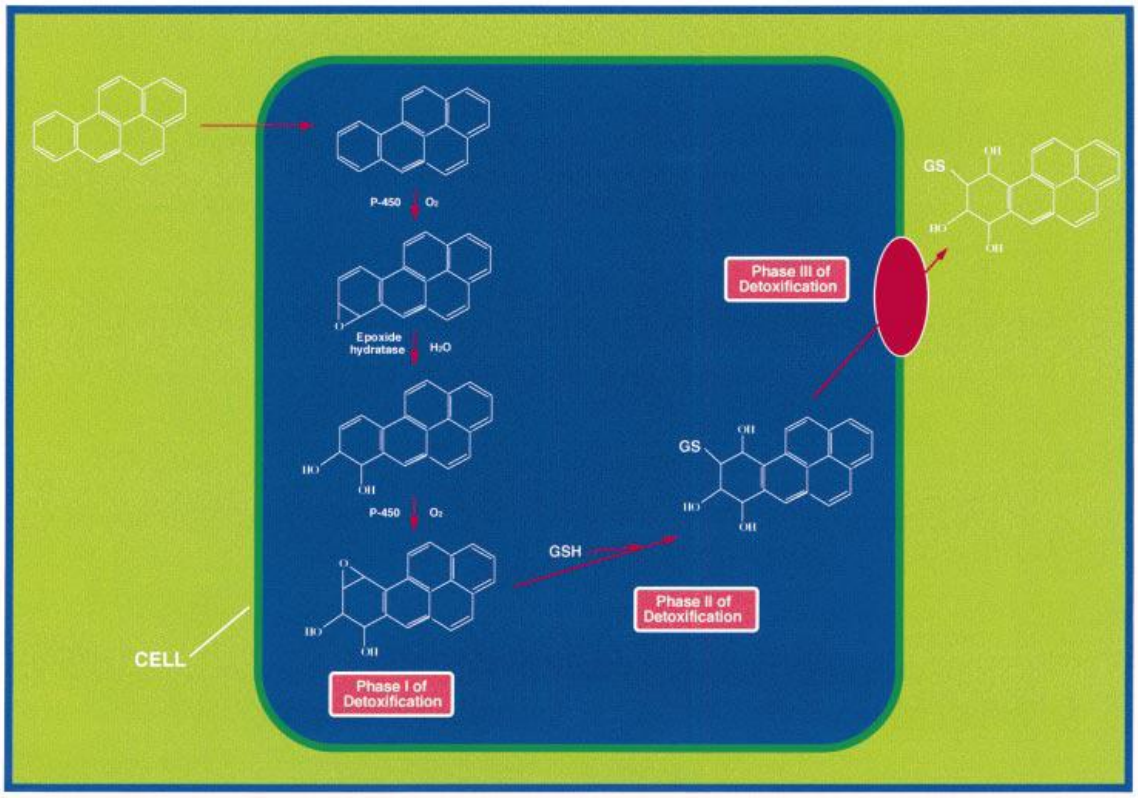

Figure 2.1 : Illustration of the possible fate of carcinogenic xenobiotic, benzo(a)pyrene, once it enter cell. Cytochrome P450 and epoxide hydratase responsible for the phase I detoxification system while GSTs are the phase II enzyme which catalyze conjugation of GSH with xenobiotic. GSH-xenobiotic conjugate is hydrophilic. Thus, it will be pumped out from the cell and eventually excreted out as mercapturic acids. (Adapted from Sheehan *et al*., 2001)

2.1.1 CLASSIFICATION OF GLUTATHIONE S-TRANSFERASES

GSTs are superfamily enzymes that have been found in most of the life form such as animals, plants, insects, helminthes, fungi and bacteria (Grover and Sims, 1964; Yu *et al*., 2003; Fakae *et al*., 2000; Enayati *et al*., 2005; Sato *et al*., 2009; Piccolomini *et al*., 1989). Candidates representing non-mammalian GSTs are such as the fungal-specific GST, GstB, which was identified and characterized by Sato *et al*. (2009); the basic GST form B which shown no homology to mammalian GST, and the GST form AI which exhibit mammalian alpha-family-like biochemical properties, were found in nematode cytosol (Papadopoulos *et al*., 1989); PfGST, which is the only isoform present in malarial parasite, shown significantly different structure of binding site compared to human isozymes (Hiller *et al*., 2006); the rho-class GST which specific for teleost fish with no homologues in mammals (Liang *et al*., 2007); the four major classes of plant GSTs which is theta, zeta, phi and tau. The theta and zeta classes have related to mammalian GST while the phi and tau classes are unique for plant GST (Wagner *et al*., 2002); and the insect GSTs which has been grouped into three classes, that is, I, II and III. Class I GSTs are insect-specific delta class and class II GSTs are members of sigma class (Agianian *et al*., 2003).

Mammalian GSTs are consists of three distinct family, that is, the cytosolic, mitochondrial and membrane-bound microsomal GSTs family (McIlwain *et al*., 2006). The dimeric cytosolic GSTs has been classified into at least eight major classes, that is, Alpha, Mu, Pi, Sigma, Theta, Kappa, Omega and Zeta based on the combination of physical, chemical, immunological, structural and catalytic properties (Mannervik *et al*., 1985; Pemble *et al*., 1996; Sheehan *et al*., 2001; Kitteringham *et al*., 2007). Meanwhile, the homotrimeric mirosomal GSTs has been classified as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Sun and Morgenstern, 1997;

Sheehan *et al*., 2001). Table 2.1 shows some examples of classification criteria for GSTs, and Figure 2.2 demonstrating the subunit structures of different GST class.

Table 2.1 : Classification criteria for GSTs

Criteria	Example of GST
Primary structure comparison	Alpha, Mu, Pi, Theta, Kappa, Zeta, Omega classes
Immunoblotting	Alpha, Mu, MIF (migration-inhibitory-factor-like) classes, Insect classes I and II, *Faciola hepatica* GSTs
Kinetic properties: Substrate specificity/affinity Inhibitor sensitivity	 Alpha, Mu, Pi, Theta classes Alpha, Mu, Pi classes
Tertiary structure: active site	Alpha, Mu, Pi, Theta, Omega, Beta, Sigma classes
Quarternary structure: Ability to hybridize into dimers Inter-subunit interface	 Mu, Alpha classes Hydrophobic lock and key in Alpha, Mu, Pi, Theta classes, Polar interface in Beta class

(Adapted from Sheehan *et al*., 2001)

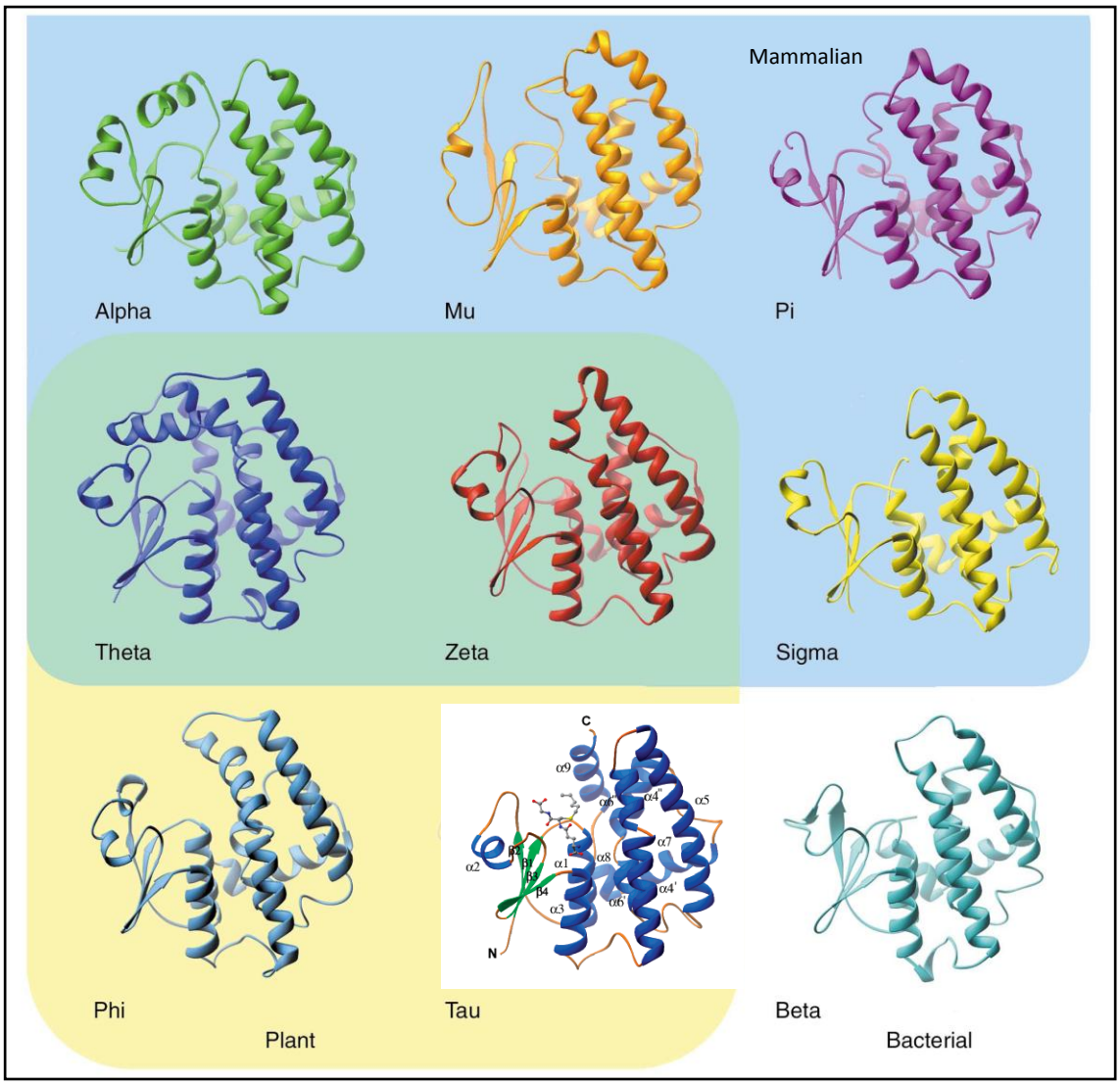

Figure 2.2 : Ribbon representations of the structures of different classes GST subunits. (Adapted from Dixon *et al*., 2002; Thom *et al*., 2002)

2.1.2 GLUTATHIONE

Glutathione (GSH) is a tripeptide of γ-glutamyl-cysteinyl-glycine, which involved in cellular defense mechanism of living organism against reactive oxygen species (ROS) and xenobiotics. GSH present freely or bound to proteins in cells. Synthesis and degradation of GSH are regulated by the reactions in γ-glutamyl cycle (Pastore *et al*., 2001). The reversible oxidation of the thiol/thiolate group of cysteine residue generates oxidized glutathione (GSSG) and acts as a major cellular redox buffer. GSH provides electron to glutathione peroxide which reduces hydrogen peroxide to water and thus contributed to ROS degradation. Oxidized GSH is then re-reduced by glutathione reductase using NADPH as an electron donor (Sato *et al*., 2009). Measurement of the ratio of reduced glutathione to oxidized glutathione in the cells used as an indicator of cellular toxicity.

Another role of GSH in cellular detoxification mechanism is regulated by GSTs. GSTs catalyzed the nucleophilic attack of glutathione sulphur atom on the electrophilic region of various xenobiotics and endogenous ROS compounds, resulting in the production of hydrophilic conjugates which are further metabolized to mercapturic acid and then excreted out of the cell (Zhang and Fang, 1999). For example, human GSTA1-1 and GSTA2-2 attenuates lipid peroxidation by catalyzing GSH-dependent reduction of phospholipid hydroperoxides and thus protect the cells from hydrogen peroxide-induced apoptosis (Yang *et al*., 2001).

Besides, GSH also involves in other cellular mechanism such as inhibition of melanin synthesis and agglutination by interrupting the function of L-DOPA (Matsuki *et al*., 2008); involvement in iron metabolism by its requirement, together with mitochondrial inner-membrane ATP binding cassette (ABC) transporter Atm1 and intermembrane Erv1 thiol oxidase in iron-sulphur cluster (ISC) assembly, and served as

a backup of thioredoxin in cytosolic thiol-redox control (Kumar *et al*., 2011); synthesis of phytochelatins (PC) which catalyzed by PC synthase in the presence of heavy metals (Ha *et al*., 1999); enhancement of humoral immune response as glutathione is an essential element for lymphocyte proliferation (Bounous *et al*., 1989); Protect DNA through donation of hydrogen atom to highly reactive hydroxyl radicals during exposure to radiation, and chemical repairment (Bounous and Gold, 1991); regulation of cell cycle, in which de novo synthesis of GSH is required for cell proliferation and proper transition of S and G2 phase (Poot *et al*., 1995); Post-translation modification of intracellular proteins by glutathionylation which changed the protein folding into different conformation (Townsend *et al*., 2003).

GSH has been reported as a critical factor in protecting living organism from diseases. Deficiency of GSH leads to neurological disorders such as impairment of mitochondria and ischemia in brain, Parkinson's disease, Alzheimer's disease, Huntington disease and Schizophrenia (Dringen, 2000).

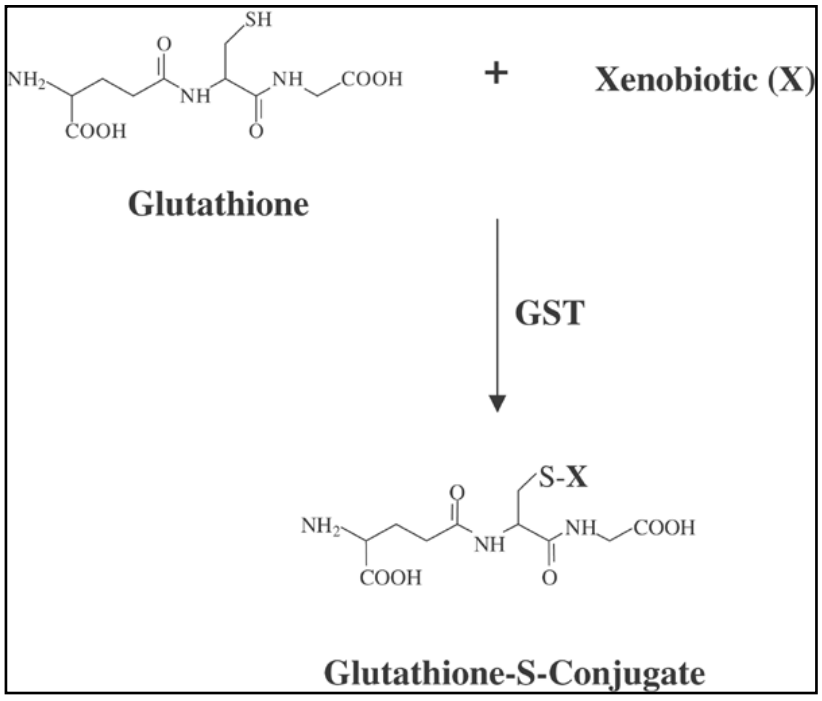

Figure 2.3 : Illustration of the conjugation of glutathione (GSH) to xenobiotic by glutathione S-transferase (GST). (Adapted from Townsend and Tew, 2003)

2.2 GLUTATHIONE S-TRANSFERASE π

Glutathione S-Transferase π (GST-P) is one of the classes of GST encoded by seven exons of polymorphic GSTP1 gene localized in chromosome 11. The GSTP1 gene has a series of single nucleotide polymorphisms (SNPs) that yield polymorphism in the amino acid sequence of encoded protein. There are 4 functionally active polymorphisms of GSTP1 have been identified (McIlwain *et al*., 2006). GST-P has a molecular weight of 24 kDa and a pI value of 7.1 (Aliya *et al*., 2003; Kitteringham *et al*., 2007). GST-P expression is inducible by cytotoxic drugs and it was found to be different from the ordinary GST subunits.

Table 2.2 : Genetic variation of GST-P

Allele	Nucleotide variability
GSTP1*A	Ile 105; Ala 114
GSTP1*B	Val 105; Ala114
GSTP1*C	Val 105; Val 114
GSTP1*D	Ile 105; Val 114

(Adapted from McIlwain *et al.*, 2006)

GST-P was initially identified as an anionic protein in human placenta, but later on it was found and characterized for most human cancer cells, such as human M-7609, Caco-2 and HT-29 colorectal cancer cells (Niitsu *et al*., 1998; Peters and Roelofs, 1989; Tashiro *et al.*, 2001), diffuse large B-cell lymphomas (Ribrag *et al*., 2003), malignant ovarian tumor cells (Satoh *et al*., 2001), renal UOK 130 cancer cells (Wang *et al*., 2007), Adr^R MCF-7 breast cancer cells (Batist *et al*., 1986), HOS osteosarcoma cell line (Huang *et al*., 2007), cutaneous squamous cell carcinomas and normal skin cell (Shimazu *et al*., 1995), stomach, gastric and lung carcinomas (Tsutsumi *et al*., 1987; Qin *et al*., 2002; Eimoto *et al*., 1988). Among all of the GST classes that have been

characterized, GST-P has significance in cancer diagnosis as it was expressed ubiquitously and abundantly in maglinant cells (Aliya *et al*., 2003).

Elevated GST-P expression is associated with multidrug resistance, malignant transformation and decreased number of estrogen receptors in breast cancer (Moscow *et al.*, 1989). GST contributes to chemoresistance in many cancers and become a major reason of chemotherapy failure and disease recurrence (Huang *et al*., 2007). Maglinant transformation associated with GST-P expression has been demonstrated in eusophagus cancer as the GST-P has been prominently expressed in esophageal squamous mucosa and adenocarcinoma (Chandra *et al*., 2002).

In addition, GST-P also regulates activities of several cellular proteins such as c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinases (p38 MAPK), Extracellular signal-regulated kinase (ERK) and TNF receptor-associated factor 2 (TRAF2) by protein-protein interactions (Adler *et al*., 1999; Adler and Pincus, 2004; Lu *et al*., 2004; Wu *et al*., 2006). Under stressed condition, the JNK inhibitor, GST-P, undergoes oligomerization and dissociated from JNK which eventually lead to the activation of JNK pathway (Adler *et al*., 1999). Lu *et al*. (2004) reported dual effects of GST-P when prostate cancer cells exposed to arsenic trioxide, As_2O_3. At low concentration of As_2O_3, GST-P enhanced cell growth inhibition by apoptosis-independent pathway, which is restricted the cell growth at G2/M phase. Conversely, GST-P inhibited As_2O_3-induced apoptosis by inhibiting the activation of JNK and p38 kinase, at high concentration of As_2O_3.

GST-P also potentiate S-glutathionylation, an alternative to nitration and nitrosylation on nitric oxide (NO), by induces nitrosative stress which results in elicits toxicity in vitro and in vivo (Townsend *et al*., 2006).

Expression of GST-P in normal cells may play a protective role in preventing chemical carcinogenesis as almost 80% of human cancer cases reported were related to environmental chemical carcinogens (Zhang and Fang, 1999). Shimazu *et al*. (1995) has reported that GST-P distributed mainly in the upper layers cell of normal epidermis and GST-P is involved in carcinogenesis process. Furthermore, down-regulation of GST-P expression has been shown to increase the risk of developing prostate and colon cancer (Okino *et al*., 2007; Ritchie *et al*., 2009). Zhang and Fang (1999) showed that GST-P transfected NIH3T3 cells expressed exogenous GST-P which protects the cells from carcinogen, glycidyl methacrylate, thus provides the contribution of GST-P in preventing chemical carcinogenesis.

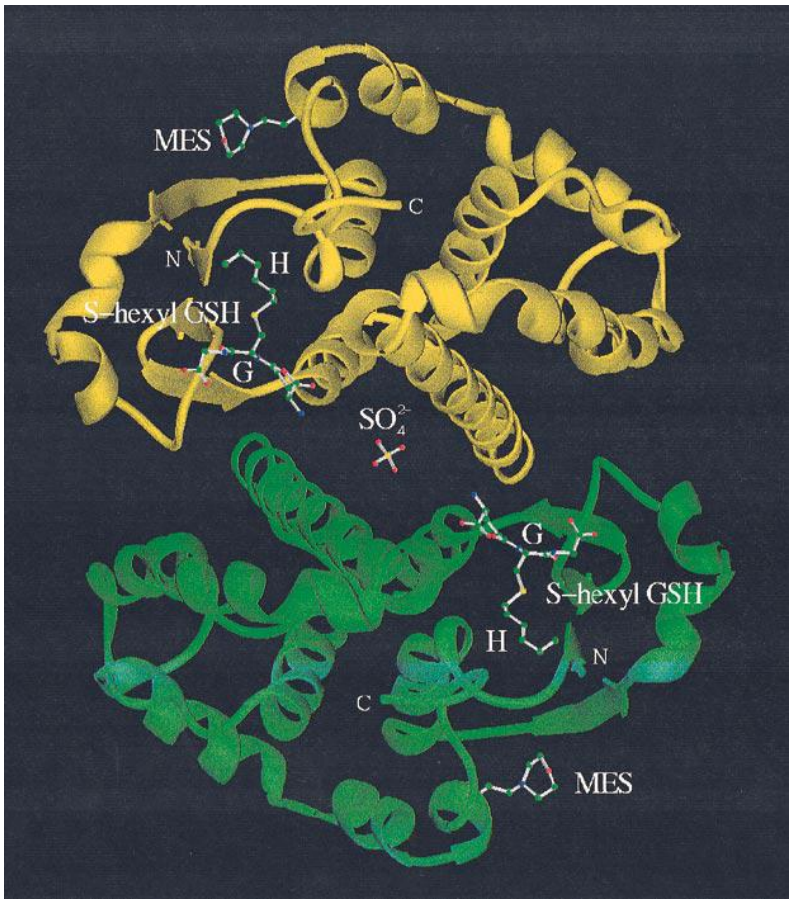

Figure 2.4 : Ribbon representation of the human GST-P dimer complexes with S-hexyl GSH. (Adapted from Oakley *et al*., 1997)

2.2.1 MULTIDRUG RESISTANCE

Acquired or intrinsic multidrug resistance is one of the mechanisms contributing to the failure of lasting chemotherapy treatment in cancer patients. Multidrug resistance during cancer therapy has been related to the high expression of GST-P. Niitsu *et al.* (1998) proved the involvement of GST-P in drug resistance by transfer of antisense gene into colon cancer cells which had high expression of GST-P and the result shows that transfectants have higher sensitivity towards alkylating agents compared to the parental cells. Moscow *et al*. (1989) shown that GST-P expression vector transfected drug-sensitive MCF-7 human breast cancer cells have elevated GST-P level and the transfectants were more resistant to cytotoxic agents. In addition, GST-P was worked as the determinant of drug resistance in GST-P transfectant cell line, pT22-3, whereby the increase in resistant toward cytotoxic agents, ethacrynic acid and adriamycin (Nakagawa *et al*., 1990). Tashiro *et al*. (2001) reported that the expression of GST-P reduced the cytotoxicity of potent cytocidal, glutathione-doxorubicin conjugate (GSH-DXR) against tumor cells and decreased GSH-DXR-induced activation of apoptotic marker, caspase-3. An earlier study also shows that GST-P gene transduced human stem cells, CD34+ cells, were resistant to alkylating agents, cyclophosphamide and adriamycin (Kuga *et al*., 1997).

Even though resistance of cancer cell GST-P towards alkylating agents in chemotherapy is affecting the results of the therapy, the strategy of utilizing GST-P gene for chemoprotection of bone marrow from subsequent high dose chemotherapy is promising for cancer gene therapy (Niitsu *et al.*, 1998). This is further supported by Matsunaga *et al.* (2000) whereby the transplantation of GST-P transduced bone marrow cells into mice conferred resistance to bone marrow of recipient mice against cyclophosphamide in high dose chemotherapy.

Besides of GST-P, there are other parameters which are implicated in multidrug resistance, such as rapid drug efflux by upregulation of ATP binding cassette (ABC) transporters expression (Mann *et al*., 1990, Sarkadi *et al*., 2006), elevated levels of detoxifying protein molecules like GSH and metallothioneins (Friesen *et al*., 2004; Kelley *et al*., 1988), overexpression of glycoprotein and enhanced DNA repair (Johnson *et al*., 1993). Figure 2.5 demonstrated different ways of drug resistance in cell when it exposed to cytotoxic drugs.

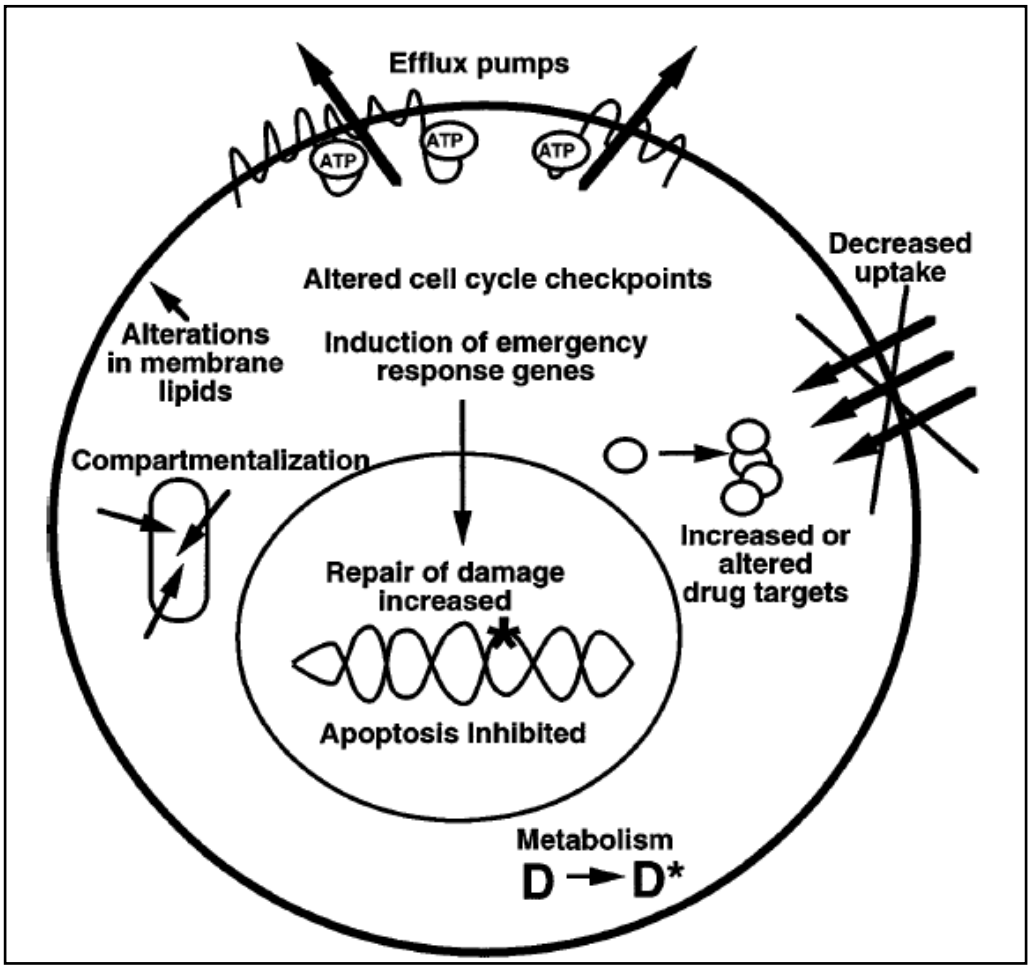

Figure 2.5 : A summary of the mechanisms of cytotoxic drugs resistance in cultured cancer cells. (Adapted from Gottesman, 2002)

2.3 GLUTATHIONE S-TRANSFERASE INHIBITORS

Several researches have been done to identify the inhibitors of GST. The importance of GST is not only concerned for cancer therapy, but also highlighted in the establishment of chronic parasite infections. Noticeable GST activity has been detected in human, rodent and simian malarial parasites. Antimalarials such as hemin, chloroquine, artemisinin and primaquine were inhibited the GST activity in a concentration-dependent manner (Srivastava *et al*., 1999). Malarial parasite PfGST which has the highest sequence similarities with GST-P was reported to be inhibited by cibacron blue, S-hexylglutathione, hemin, protoporphyrin IX and ferriprotoporphyrin IX (Harwaldt, *et al*., 2002; Tripathi *et al*., 2007). When Ahmad and Srivastava (2007) treated hemin on purified GST of rodent malarial parasite, *Plasmodium yoelii*, noncompetitive inhibition kinetic was observed. Hemin is also a known inhibitor of human GST. On the other hand, Triclabendazole ($C_{14}H_9Cl_3N_2OS$) was reported to have inhibitory effect on nematodirus, *Fasciola* spp. and sheep liver tissue GST (Farahnak *et al*., 2006; 2007; Seyyedi *et al*., 2005). Hexachlorophene ($C_{13}H_6Cl_6O_2$) was also found to be the inhibitor of helminth and sheep liver tissue GST but with higher affinity toward helminth GST (Farahnak and Brophy, 2004). There are also several traditional Nigerian medicinal plants found to have inhibition activity on nematode GST and thus the pharmacological basis of these plants used traditionally to treat gastrointestinal helminth infection is revealed (Fakae *et al*., 2000). In addition, Brophy *et al*. (2000) synthesized a series of β-carbonyl substituted glutathione conjugates to inhibit OvGST which topological structure closely related to mammalian GST-P in the form of wide hydrophobic binding cleft. Selectivity of the glutathione conjugates for OvGST over human GST-P is reported to be higher than 10-fold. Od-GST of porcine nodule nematode, *Oesophagostomum dentatum*, was found to be functionally similar to prostaglandin D synthase and reversibly inhibited by sulphobromophthalein,

indomethacin and ethacrynic acid. Sulphobromophthalein is known to be a specific inhibitor of alpha and pi-classes of GST (Joachim *et al*., 2011).

Other than helminth GST inhibitors, Bromosulphophtalein, cibacron blue, indomethacin, S-hexylglutathione, tributyltin acetate and rose bengal have been characterized as the inhibitors of rat liver microsomal glutathione S-transferase (Mosialou and Morgenstern, 1990; Ji *et al*., 1996), while bile acids, propylthiouracil, S-oxides of propylthiouracil, acetonitrile and haloacetonitriles have been demonstrated to have inhibition activity on rat liver cytosolic GSTs (Vessey and Zakim, 1981; Kariya *et al*., 1986; Ahmed *et al*., 1989). Wu and Mathews (1983) work on Indomethacin (1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid) inhibition of rat liver GSTs indicates that the compound noncompetitively inhibited GST conjugation with 3,4-dichloronitrobenzene (DCNB) but uncompetitively inhibited the conjugation with glutathione. On the other hand, meclofenamic acid was found to be a competitive inhibitor of GSTs. Danielson and Mannervik (1988) further reported rat liver mu-class GST (GST-M) was competitively inhibited by micromolar concentration of indomethacin in a condition of high CDNB concentration. Interestingly, the inhibition pattern of indomethacin on GST-M seems to be paradoxical when CDNB used as a substrate. Early on, Mitra *et al.* (1991) shown that polyunsaturated fatty acids (particularly arachidonic acid), and saturated fatty acid esters (such as ascorbate) caused remarkable inhibition activity on rat and mouse liver GST. After that, they noticed that saturated fatty acids and ascorbate esters of saturated fatty acids also caused significant inhibition on human placental and liver GST, especially for the GST-P. Reversible competitive inhibition of ascorbyl stearate on placental GST was suggested based on the evidence found (Mitra *et al*., 1992). Acetylenic fatty acids such as 5,8,11-eicosatriynoic acid (ETI) and 5,8,11,14-eicosatetraynoic acid (ETYI), were also reported to cause inhibition of human and rat hepatic GST with substrate CDNB. Kinetic study revealed

noncompetitive inhibition of ETI on human hepatic GST (Datta and Kulkarni, 1994). Dicumarol, an anticoagulant analogue of vitamin K obtained from sweet clover, was demonstrated to cause inhibition of several classes of mouse GST. It inhibited mouse hepatic GST-M to conjugate with CDNB. With substrate 4-nitroquinoline 1-oxide (4-NQO), mu and pi classes of GST were inhibited, whereas with cumene hydroperoxide (CHP), GSTA is the one been inhibited. Besides of GST, Dicumarol used to be a specific inhibitor of DT-diaphorase (NAD(P)H: quinine oxidoreductase) (Mays and Benson, 1992). In addition, reversible and irreversible inhibition of rat GSTs by caffeic acid and its 2-S-glutathionyl conjugate were demonstrated by Ploemen *et al*. (1993c).

Table 2.3 : GST inhibitors

Type of GST	**Inhibitor**	**Ki**	**IC_{50} value**	**Reference**
Plasmodium yoelii GST	Hemin	4 µM	4 µM	Ahmad and Srivastava, 2007
Plasmodium yoelii GST	Protoporphyrin IX	13 µM	12.5 µM	Ahmad and Srivastava, 2008a
	Cibacron blue	0.4 µM	56.3 µM	
	Menadione	80 µM	> 200 µM	
Plasmodium falciparum PfGST	Cibacron blue	0.5 µM	-	Harwaldt *et al.*, 2002
	S-hexylglutathione	35 µM	-	
	Protoporphyrin IX	10 µM	-	
	Hemin	6.5 µM	-	
	Chloroquine	-	> 200 µM	
Plasmodium falciparum PfGST	Ferriprotoporphyrin IX	1.4 µM	1 µM	Liebau *et al*., 2002
Fasciola hepatica GST	Hexachlorophene	-	0.25 µM	Farahnak and Brophy, 2004
Sheep liver GST	Hexachlorophene	-	1 µM	
Ascaris suum GST	*P. thonningii* extract	-	2 mg/L	Fakae *et al*., 2000
	O. gratissimum extract	-	10 mg/L	
	N. latifolia extract	-	15 mg/L	
Onchocerca volvulus GST	*P. thonningii* extract	-	4 mg/L	
	O. gratissimum extract	-	8 mg/L	
	N. latifolia extract	-	28 mg/L	
Oesophagostomum dentatum Od-GST	Sulphobromophthalein	-	150 µM	Joachim *et al*., 2011
	Indomethacin	-	56-280 µM	
	Ethacrynic acid	-	0.05-5 µM	
	HQL-79	-	330 µM	
Caenorhabditis elegans GST	Sulphobromophthalein	-	17 µM	
Schistosoma mansoni Sm28 GST	Sulphobromophthalein	-	0.09 µM	
Schistosoma japonicumi GST	Sulphobromophthalein	-	36.4 µM	
Setaria digitata GST	Ethacrynic acid	-	19.42 µM	
Setaria cervi GST	Hemin	4 µM	25 µM	Ahmad and Srivastava, 2008b
	Ethacrynic acid	22 µM	15 µM	
	S-hexylglutathione	0.04 mM	0.1 mM	
	Quercetin	5 µM	25 µM	
	Cibacron blue	0.02 µM	0.025 µM	
	Lithocholate sulfate	0.1 mM	0.3 mM	
	Ellagic acid	0.27 µM	0.8 µM	
Rat liver GST	Indomethacin	53 µM, with DCNB	-	Wu and Mathews, 1983
	Indomethacin	40 µM, with GSH	-	
	4-Chlorobenzoic acid	470 µM, with DCNB	-	
	5-methoxy-2-methylindole-3-acetic acid	330 µM, with DCNB	-	

Table 2.3, continued

Type of GST	Inhibitor	Ki	IC_{50} value	Reference
Rat liver GST	Meclofenamic acid	300 μM, with DCNB	-	Wu and Mathews, 1983
Rat liver cytosolic GST	Acetonitrile	-	> 10 mM	Ahmed *et al*., 1989
	Monofluoroacetonitrile	-	> 10 mM	
	Monochloroacetonitrile	-	> 10 mM	
	Monobromoacetonitrile	-	> 10 mM	
	Dichloroacetonitrile	-	2.49 mM	
	Trichloroacetonitrile	-	0.34 mM	
	Dibromoacetonitrile	-	0.82 mM	
	Monoiodoacetonitrile	-	4.44 mM	
Rat liver microsomal GST	Indomethacin	-	800 μM	Ji *et al.*, 1996
Human placental GST	Ascorbyl stearate	3.1 μM	-	Mitra *et al*., 1992
	Ascorbyl palmitate	10.0 μM	-	
	Palmitic acid	13.5 μM	-	
	Stearic acid	18.5 μM	-	
Human fetal liver GST	Ascorbyl stearate	-	15 μM	
	Ascorbyl palmitate	-	45 μM	
	Palmitic acid	-	83 μM	
	Stearic acid	-	78 μM	
Rat liver GST	Ascorbyl stearate	-	21 μM	
	Ascorbyl palmitate	-	6 μM	
	Palmitic acid	-	88 μM	
	Stearic acid	-	117 μM	
HT-29 GST	Ethacrynic acid	-	30 μM	Tew *et al.*, 1988
	Piriprost	-	22 μM	
BE GST	Ethacrynic acid	-	30 μM	
	Piriprost	-	170 μM	
Rat GST-M	Indomethacin	-	1 μM	Danielson and Mannervik, 1988
Rat GSTA 1-1	Caffeic acid	-	> 640 μM	Ploemen *et al*., 1993c
	2-S-glutathionylcaffeic acid	-	13 μM	
Rat GSTA 2-2	Caffeic acid	-	> 640 μM	
	2-S-glutathionylcaffeic acid	-	> 125 μM	
Rat GST-M 3-3	Caffeic acid	-	360 μM	
	2-S-glutathionylcaffeic acid	-	7.1 μM	
Rat GST-M 4-4	Caffeic acid	-	58 μM	
	2-S-glutathionylcaffeic acid	-	26 μM	
Rat GST-P 7-7	Caffeic acid	-	470 μM	
	2-S-glutathionylcaffeic acid	-	36 μM	

Table 2.3, continued

Type of GST	Inhibitor	Ki	IC_{50} value	Reference
Mouse GST-M	Dicumarol	-	11 µM, with CDNB	Mays and Benson, 1992
	Dicumarol	-	14 µM, with 4-NQO	
Mouse GST-P	Dicumarol	-	9 µM, with 4-NQO	
Mouse GSTA	Dicumarol	-	14 µM, with CHP	
Rat and human GST-M	Dibromodihydroethacrynic acid	-	0.4-0.6 µM	Ploemen *et al*., 1993a
Rat and human GST-P	Dibromodihydroethacrynic acid	-	4.6-10 µM	
Rat and human GSTA	Ethacrynic acid	-	4.6-6.0 µM	Ploemen *et al*., 1993b
	Glutathione-Ethacrynic acid conjugate	-	0.8-2.8 µM	
Rat and human GST-M	Ethacrynic acid	-	0.3-1.9 µM	
	Glutathione-Ethacrynic acid conjugate	-	< 0.1-1.2 µM	
Rat and human GST-P	Ethacrynic acid	-	3.3-4.8 µM	
	Glutathione-Ethacrynic acid conjugate	-	11 µM	
RPMI 8322 GSTA	Ethacrynic acid	-	10 µM	Hansson *et al*., 1991
RPMI 8322 GST-M	Ethacrynic acid	-	1 µM	
RPMI 8322 GST-P	Ethacrynic acid	-	15 µM	
Human GST-P	TER 117	0.4 µM	-	Morgan *et al*., 1996
	TER 135	0.85 µM	-	
	TER 211	1.2 µM	-	
	TER 143	1.9 µM	-	
	Ethacrynic acid	4.0 µM	-	
Human GSTA	TER 117	20 µM	-	
	TER 135	5.8 µM	-	
	TER 211	4.2 µM	-	
	TER 143	0.27 µM	-	
	Ethacrynic acid	2.0 µM	-	
Human GST-M 1a-1a	TER 117	25 µM	-	
	TER 135	41 µM	-	
	TER 211	0.01 µM	-	
	TER 143	1.2 µM	-	
	Ethacrynic acid	3.0 µM	-	
Human GST-M 2-2	TER 117	31 µM	-	
	TER 135	97 µM	-	
	TER 211	1.5 µM	-	
Human GST-P	Hypochlorous acid	0.55 µM	0.6 µM	van Haaften *et al*., 2001
Human GST-P	Auranofin	-	32.9 µM	De Luca *et al*., 2012
Human GST-P	Ethacrynic acid	-	3.4 µM	Yang *et al*., 2010
	Ethacrynic acid oxadiazole analogs	-	0.6-5.4 µM	

2.3.1 GST-P INHIBITOR

To counteract the phenomenon of multidrug resistance which partly caused by the GST-P, potential inhibitors for this enzyme has been found and designed to sensitize those tumor cells to antineoplastic drugs. Ethacrynic acid (EA), which originally found as plant phenolic acid, is reported to be preferentially conjugated by alpha, mu and pi-class GSTs (Ploemen *et al*., 1993b; Liebau *et al*., 2002). Reversible inhibition of GSH-EA conjugates on alpha and mu-classes of GST is reported to be more potent than EA itself, but not for GST-P (Ploemen *et al*., 1993b). However, dibromo dihydro derivative of EA exhibit strong inhibitory capacity on both human and rat GST-P (Ploemen *et al*., 1993a). EA has been used to increase the sensitivity of tumor cells towards alkylating cytostatic agents used in chemotherapy. EA enhanced the cytotoxic effects of chlorambucil in patients with chronic leukemia and also in human colon carcinoma cell line in *in vitro* assays. Besides, EA has been found to potentiate the effect of melphalan in human colon tumor xenografts of SCID mice (Townsend and Tew, 2003). According to Tew *et al*. (1988), EA and piriprost (6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1) have the ability to enhance cytotoxic activity of chlorambucil on rat and human tumor cells by interfering thiol metabolism and GST functions. Both of them have shown significant inhibitory activity on intracellular GST which result in the reversion of drug-resistant cells to the drug-sensitive cells. Even though EA is a potent GST-P inhibitor, EA is also both inhibitor and inducer of GST which found to increases the expression of GST-P and also half-life of the protein (Shen *et al.*, 1995). Non-specific inhibition of EA on GST isozyme and also side effects limited its clinical application as the possible deleterious effects on normal cell's defense system should also take into consideration (Wang *et al*., 2008).

A potent and specific inhibitor of GST-P has been developed by Schultz *et al.* (1997), which is the glutathione analog Terrapin 199 (TER 199). Later on, TER 117 has

been developed for efficient inhibition of the most abundant form of GST-P/Ile 105. It was found that the inhibitory effect of TER 117 on both GST-P and glyoxalase I may act synergically and thus improve the effectiveness of chemotherapy. TER 117 is a GSH analog designed to block the active site of GST-P (Johansson *et al*., 2000).

The inhibition of GST-P by TLK 199 results in the activation of c-Jun NH_2-terminal kinases (JNK) thus induced cell apoptosis. TLK 199 is a rationally designed GSH-based-peptidomimetic inhibitor of GST-P (Townsend and Tew, 2003). It has been reported as an inhibitor of the multidrug resistance-associated protein-1 (MRP-1) which reversed the resistance to various types of chemotherapeutic agents in NIH3T3 cells which transfected with MRP-1 (O'Brien *et al*., 1999).

A GST-P specific inactivator, haloenol lactone (HEL) derivative, was designed by Zheng *et al*. (1996). HEL is a site-directed inhibitor targeted on the nucleophile near the active site of GST. Binding of HEL to the nucleophile leads to opening of lactone ring, results in the formation of α-bromoketone intermediate which permanently modified the protein. HEL enhanced cytotoxicity of alkylating agents by GST inhibition and GSH depletion (Wang *et al.*, 2008).

Another potent, non-competitive GST-P inhibitor, hypochlorous acid (HOCl), was introduced by van Haaften *et al*. (2001). HOCl was generated by neutrophils through the conversion of H_2O_2 by myeloperoxide. HOCl inhibited GST-P with an IC50 value thousand times lower than IC50 value reported for H_2O_2 previously. H_2O_2 is ROS that specifically inhibited GST-P but has no inhibition effect on other GST subclass, such as Mu-class GST. Even though GSTs were known to be the class of enzyme to combat ROS during oxidative stress, GST-P was an exception. The powerful oxidant HOCl not only capable of inhibiting GST-P but also induced apoptosis through the activation of caspase 3 (Vissers *et al*., 1999). However, uncontrolled and/or excessive

production of HOCl may leads to oxidative stress and harmful effects to surrounding cells (Li *et al.*, 2004).

Anticancer metallodrugs act on cytosolic GSTs were reported to cause potent and selective inhibition of GST-P. EA conjugated ruthenium-arene complexes are reported as one of the most effective rutherium complexes-based GST-P inhibitor (Ang *et al.*, 2007). Gold compound, auranofin, exhibit strong anticancer effects and also causes inhibition of pi-class GST. Auranofin inhibited GST-P in a way different from other reported inhibitor, where cysteine residues are not essential for enzyme inactivation (De Luca *et al*., 2012). Moreover, pharmacological mode of action of gold compounds is targeted on essential enzymes rather than DNA. For example, Gold (I) carbene complexes reported to have cytotoxic effects on cancer cell lines by causing thioredoxin 1 and thioredoxin 2 oxidation (Schuh *et al*., 2012) and recombinant human serum albumin conjugated organoruthenium complexes are shown to enhance cytotoxicity with known cyclin-dependent kinase (Cdk) inhibition activity (Stepanenko *et al*., 2011).

Structures of the GST inhibitors are shown in Figure 2.6.

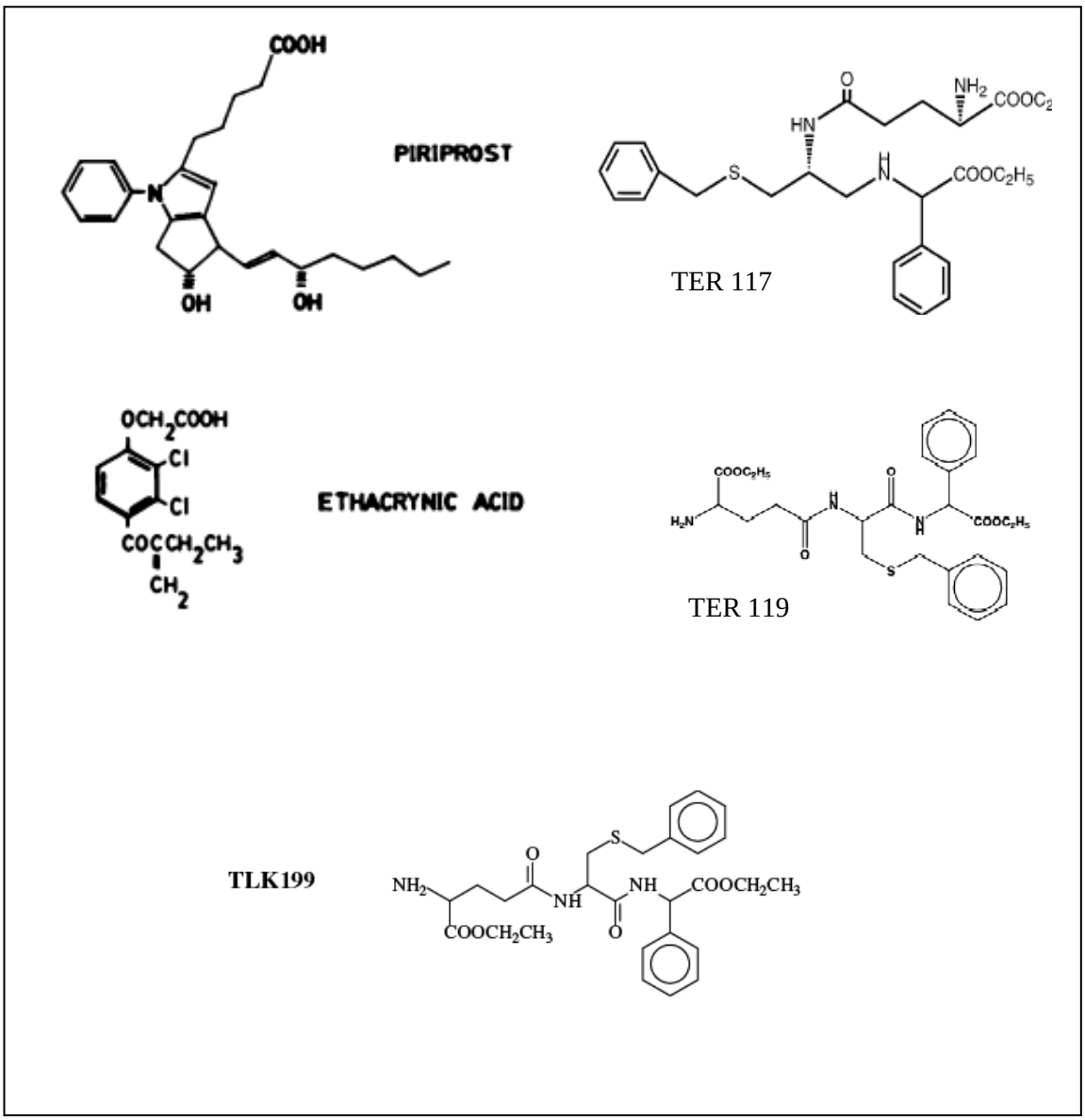

Figure 2.6 : Structures of GST inhibitors. (Adapted from O'Brien *et al*., 1999; Tew *et al*, 1988; Schultz *et al*., 1997; Townsend and Tew, 2003)

2.4 COLORECTAL CANCER

Colorectal cancer, or commonly known as colon cancer or bowel cancer, is cancer initially develop in glands in the lining of colon (large intestine) or rectum (end of colon) (Medical Encyclopedia, 2012). The colorectal cancers are adenocarcinomas which progress from normal to dysplastic epithelium to carcinoma. It involves alteration of selected gene that eventually results in abnormal rates of apoptosis and proliferation (Evans *et al*., 2006). Most of the colorectal cancer developed through the formation benign polyp on the inner lining (mucosa) of the colon or rectum (Lanza *et al*., 2011).

In order to choose an appropriate treatment for the disease, determination of the colon cancer stage becomes a very important part of diagnosis. Basically, colon cancer can be divided into 5 different stages.

Stage 0: Also known as carcinoma *in situ*, where the cancerous cells are limited at the inner lining (mucosa layer) of colon.

Stage 1: Cancer is formed in the mucosa layer and spread out to the submucosa and muscle layers of colon.

Stage 2: Cancer has spread through the muscle layer to the outermost layer (serosa layer) of colon. The cancer will then spread through the serosa layer followed by spreading to nearby organs.

Stage 3: Cancer probably spread through mucosa, submucosa and muscle layers of colon, and reach either 1-3 nearby lymph nodes or tissues surrounding the lymph nodes or just 4-6 nearby lymph nodes or more than 7 nearby nodes. The cancer is then metastasis to nearby organs.

Stage 4: At this stage, the cancer has spread through the colon wall and one or more organs at other parts of the body, such as liver, lung and ovary. The cancer metastasized through blood and lymph nodes. (National Cancer Institute, 2012)

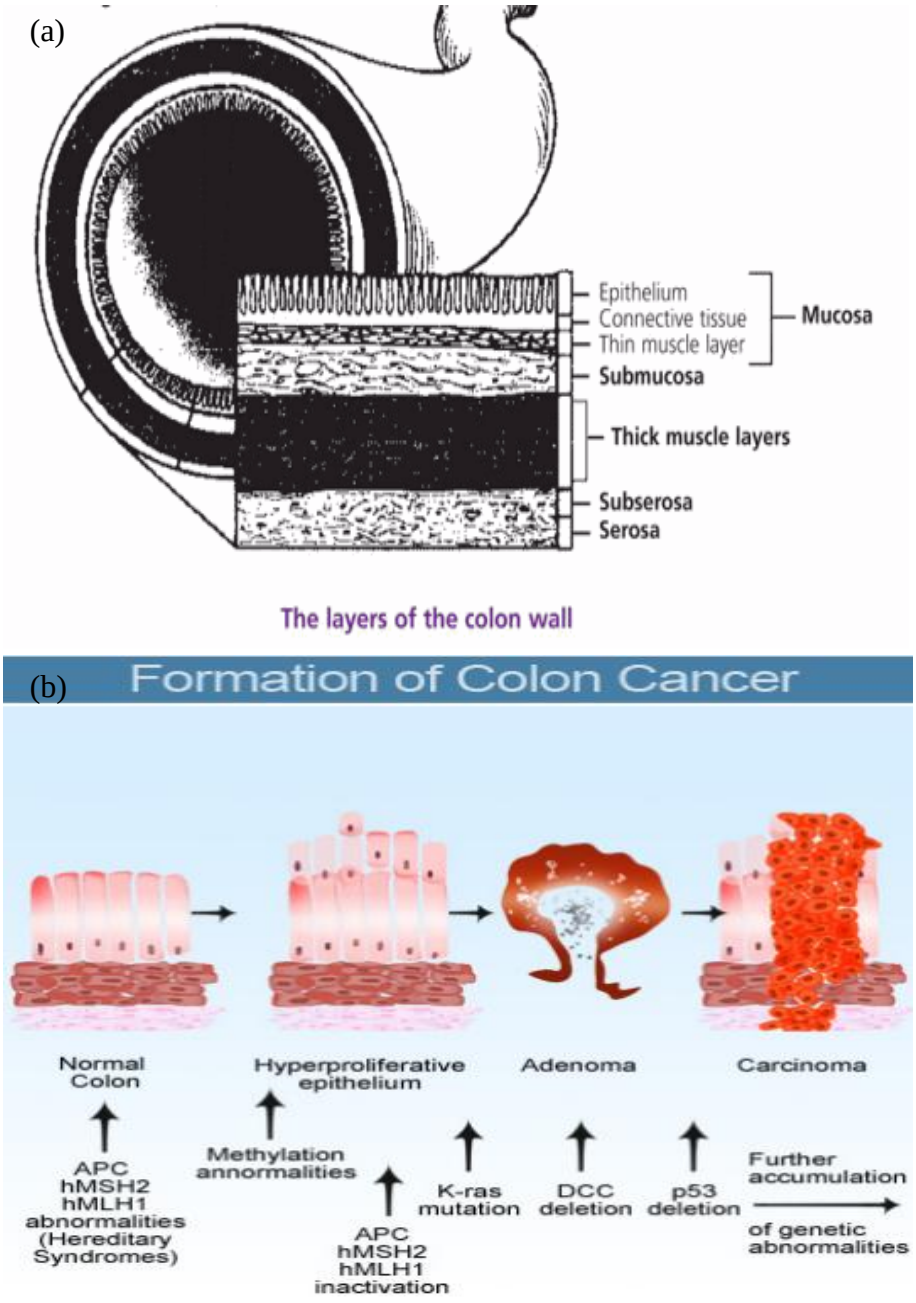

Figure 2.7 : (a) Illustration of the cross section of normal intestinal tract. (Adapted from American Cancer Society, 2012) (b) Scheme of the initiation and progression of colon cancer. (Adapted from Marks, 2012)

Colorectal cancer is one of the leading causes of death by cancer worldwide. According to the World Health Organization (WHO), there were 639,000 deaths worldwide attributed to colorectal cancer and it is the second leading cause of cancer-related death in Western country (World Health Organization, 2009). Colon cancer is reported as the second most frequent cancer in Malaysia (MAKNA, 2006). Besides, colorectal cancer is also reported as the third most diagnosed cancer in male and second most diagnosed cancer in female of England in the year 2009 (Cancer Research UK, 2012). Risk factors for development of colon cancer include gender, age, genetics, race, diet, obesity, cigarette smoking, environmental carcinogens, Lynch syndrome and polyps (Salmon and Sartorelli, 1998; Giovannucci, 2002; Pande *et al*., 2010; Lanza *et al*., 2011).

Until now, surgical resection still considered as the only curative treatment for colorectal cancer and the chances of cure is higher for earlier pathological stage (Jänne & Mayer, 2000). Since only around 50% of these cancers can be cured by either surgery or other mode of treatments, there is considerable need for further study on this kind of disease (Pfragner and Freshney, 2004). Colon cancer is relatively resistant to most of the chemotherapeutic treatments. Success of cancer chemotherapy primarily limits by cytostatic resistance (Hengstler *et al*., 1998). High level of GST-P found to be expressed in human colon cancer cell line and it is believed that GST-P indeed one of the multidrug resistance factors.

For in vitro studies to be relevant clinically, cell lines that are representative to a disease are important. A numbers of the colon carcinoma cell lines have been established and deposited at the American Type Culture Collection (ATCC) and European Collection of Cell Cultures (ECACC) cell banks. Examples of established human colorectal adenocarcinoma cell lines are such as WiDr (Noguchi *et al*., 1979), COLO 320DM (Quinn *et al*., 1979), HCT 116 (Brattain *et al*., 1981), LoVo (Drewinko

et al., 1976), Caco-2 (Jumarie and Malo, 1991), HT-29 (Chen *et al*., 1987), LS 123 (Rutzky *et al*., 1983) and BE (Tew *et al*., 1988) cell lines.

In my research, HT-29 cell line is selected for the cytotoxicity assays and extraction of GST-P enzymes as the cell line expresses high level of GST-P. In fact, HT-29 cell line was reported to be more resistant to cytotoxic agent compared to other cell lines. High GST-P-expressing HT-29 cell line has an IC_{50} value 3 times higher than the IC_{50} value of low GST-P-expressing HepG2 cells in response to doxorubicin. Toxicity of GSH-conjugated doxorubicin is higher compared to doxorubicin, where IC_{50} values for HT-29 and HepG2 are 28 and 0.15 nM, respectively (Tashiro *et al*., 2001). Felth *et al.* (2009) reported that HT-29 cells were significantly more resistant to cytotoxic cardiac glycosides and standard chemotherapeutic drugs, compared to HCT 116 and CC 20 cell lines. Monomethyltriazene is also demonstrated to be more toxic to BE cell line compared to drug-resistant HT-29 cell line (Gibson *et al.*, 1986). Furthermore, HT-29 cell line is one of the best described cell line among several adenocarcinoma cell lines and has been extensively used in the study of colorectal cancer (Lu *et al*., 1992). For example, HT-29 cell line was shown to become more resistant to methotrexate in an increasing concentration manner by growth adaptation associated cell differentiation. The morphological and functional characteristics of the differentiated phenotypes are examples of tumor heterogeneity and probably responsible for poor prognosis of colon cancers (Lesuffleur *et al.*, 1990).

2.5 ANTICANCER DRUGS

Generally, chemotherapy referred as the treatment of cancers with one or in combination of several anticancer drugs, depending on the treatment regimen. Although

there are several other cancer treatments like radiation therapy and surgery, chemotherapy still remain as the most common used treatment for solid tumors.

There are different types of anticancer drugs available nowadays, such as alkylating agents, antimetabolites, topoisomerase inhibitors, cytotoxic antibiotics, hormonal agents, plant alkaloids and miscellaneous antineoplatics. They are grouped based on factors such as mode of action, chemical structure and interaction with other drugs. Generally, the mechanisms of action of different anticancer drugs are such as following:

Alkylating agent: Prevent cell proliferation by directly damage intracellular DNA, RNA and proteins with the formation of covalent bonds. Not cell-cycle-phase-specific.

Antimetabolites: Kill cells by either interfering catalytic or regulatory activities of intracellular enzymes, or competing with metabolites to incorporate into DNA and RNA. More effective on S phase cells compared to G_0 phase cells.

Topoisomerase inhibitor: Prevent DNA synthesis by interfere with DNA topoisomerase, which functions to unwind double-stranded DNA during transcription or replication. Cell cycle is trapped at G_1 phase.

Cytotoxic antibiotic: Prevent DNA replication by intercalation of DNA and inhibition of topoisomerases. Not cell-cycle-phase-specific.

Hormonal agent: Block the supply or production of hormones needed for cancer cell growth.

Plant alkaloid: Antimitosis by inhibition of enzymes regulating cell proliferation. Most active on cell of M phase but can also act on cells in all phases. (Page and Takimoto, 2004)

Many more new drugs have been developed and tested in the attempt to achieve long lasting effect (Salmon and Sartorelli, 1998).

Table 2.4 : Different types of anticancer drugs used in cancer therapy

Types of Anticancer Drugs	**Examples**
Alkylating agents	Nitrogen mustards (Chlorambucil, Melphalan, Mechlorethamine, Ifosfamide, Cyclophosphamide), Ethylenimines (Thiotepa, Hexamethylmelamine), Alkylsulfonates (Busulfan), Hydrazines and Triazines (Altretamine, Procarbazine, Dacarbazine, Temozolomide), Nitrosureas (Carmustine, Lomustine, Streptozocin) and Metal salts (Cisplatin, Carboplatin, Oxaliplatin).
Antimetabolites	Folic acid antagonists (Methotrexate, Pemetrexed), Adenosine deaminase inhibitors (Cladribine, Fludarabine, Pentostatin), Purine antagonists (6-Mercaptopurine, 6-Thioguanine, Clofarabine) and Pyrimidine antagonists (5-Fluorouracil, Capecitabine, Cytarabine, Gemcitabine).
Topoisomerase inhibitors	Topoisomerase I inhibitors (Irinotecan, Topotecan) and Topoisomerase II inhibitors (Amsacrine, Etoposide, Mitoxantrone, Teniposide).
Antibiotics	Anthracyclines (Doxorubicin, Daunorubicin, Epirubicin, Idarubicin, Mitoxantrone), Chromomycins (Actinomycin-D, Plicamycin) and Miscellaneous (Mitomycin-C, Bleomycin).

Table 2.4, continued

Types of Anticancer Drugs	**Examples**
Hormones	Corticosteroids (Dexamethasone, Methylprednisolone, Prednisone), Antiestrogens (Fulvestrant, Tamoxifen, Toremifene), Aromatase inhibitors (Anastrozole, Letrozole, Exemestane), Estrogens, Progestins (Megestrol acetate), Antiandrogens (Bicalutamide, Flutamide, Nilutamide) and Gonadotropin-releasing hormones (Leuprolide, Goserelin).
Plant alkaloids	Vinca alkaloids (Vincristine, Vinblastine, Vinorelbine), Taxanes (Docetaxel, Paclitaxel), Podophyllotoxins (Etoposide, Tenisopide) and Camptothecan analogs (Irinotecan, Topotecan).
Immunomodulators	Monoclonal antibody (Rituximab, Alemtuzumab), Cytokines (Interferon-alfa, Interleukin-2), Vaccines (BCG, Sipuleucel-T, Oncophage) and Immunomodulating drugs (Thalidomide, Lenalidomide).
Miscellaneous antineoplastics	Adrenocortical steroid inhibitors (Mitotane), Antimicrotubule agents (Estramustine), Enzymes (L-asparaginase, Pegaspargase), Proteasome inhibitors (Bortezomid, Disulfiram, Carfilzomid), Ribonucleotide reductase inhibitors (Hydroxyurea), Retinoids (Bexarotene, Arsenic trioxide, Tretinoin) and Tyrosine kinase inhibitors (Imatinib, Gefitinib, Sunitinib)

(Adapted from American Cancer Society, 2011)

Cisplatin

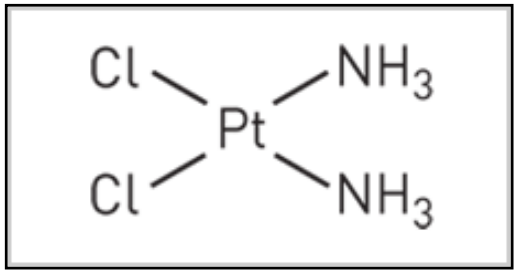

Figure 2.8 : Structure of cisplatin. (Adapted from Trzaska, 2005)

Cisplatin or *cis*-diamminedichloroplatinum(II) is one of the most potent platinum-based chemotherapy drug clinically used to treat a wide variety of tumors. It is also known as "penicillin of cancer drugs" because of its wide prescription as well as the first effective treatment for cancers (Trzaska, 2005). Cisplatin has major antineoplastic activities on sarcoma, testicular, ovarian, bladder, uterine, cervical, non-small cell and small cell lung cancer, head and neck cancer, and germ cell tumor (Page and Takimoto, 2004).

The DNA damaging effect of cisplatin is mediated by its interaction with DNA that forms intra- and inter-strand DNA adducts, which in turn activates several apoptotic pathways lead to cell apoptosis (Siddik, 2003). Cisplatin induced activation of MAPK apoptotic pathways of ERK, JNK and p38 in human ovarian carcinoma cells. ERK pathway is responsible for the cell protection against apoptosis and regulation of cell proliferation and differentiation, while JNK and p38 pathways play important roles in stress and inflammatory responses. Sustained activation of JNK and p38 pathways by cisplatin were shown to trigger up-regulation of Fas ligand expression, which accompanied by caspase induction and apoptosis (Mansouri *et al*., 2003). Besides, Pruefer *et al*. (2008) shown that cisplatin induced apoptosis in colon cancer cells by the intrinsic pathway involving Omi Htra2 serine protease. Omni Htra2 serine protease is

one of the mitochondrial releasing apoptotic proteins which inactivate inhibitors of apoptosis proteins (IAPs).

Even though cisplatin was extensively used in the treatment of solid tumor, its effectiveness is limited by the development of multidrug resistance (Parker *et al*., 2011). Most of the cisplatin-resistant cell lines showed overexpression of GST-P isozymes and reversal of drug resistance is demonstrated with the inhibition of GST-P (Pasello *et al*., 2008; Nakajima *et al*., 2003; Huang *et al*., 2007). According to Huang *et al*. (2007), GST-P expression is up-regulated when osteosarcoma cells exposed to cisplatin and this caused the cells to be more resistant to cisplatin. Both of the mRNA and protein levels of GST-P are increased in SAOS-2 cells treated with cisplatin. In contrast, GST-P suppression by gene silencing resulted in 2.5-fold increase in cell growth inhibition when treated with cisplatin. GST-P suppression increased apoptosis and DNA destruction in response to cytotoxic drugs exposure, and this also decreased the cisplatin-induced signal activation of kinase1/2 thus enhanced chemosensitivity.

The use of cisplatin is also limited by its side effects, such as nephrotoxicity, neurotoxicity, ototoxicity, myelosuppression, radiosensitizer, electrolyte imbalance, nausea, and vomiting (Page and Takimoto, 2004). However, the major dose-limiting toxicity of cisplatin is nephrotoxicity, which indicated by damage of renal tubule due to the elevation of serum creatinine or blood urea nitrogen (BUN) (Barakat *et al*., 2009).

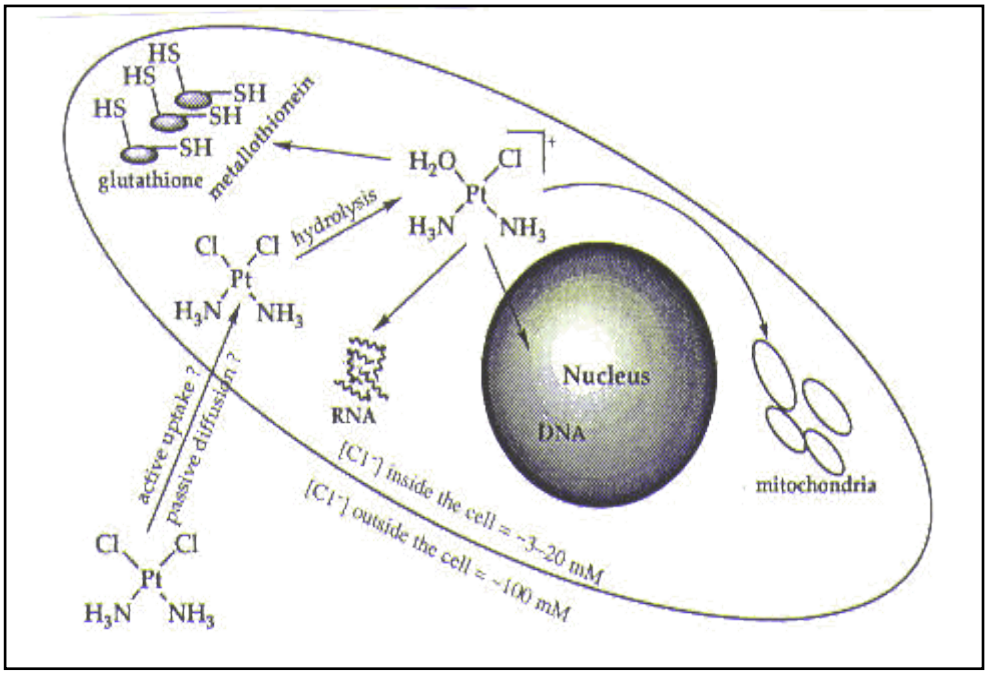

Figure 2.9 : Illustration of cellular uptake of cisplatin and its possible intracellular targets. (Adapted from Pil and Lippard, 1997)

Doxorubicin

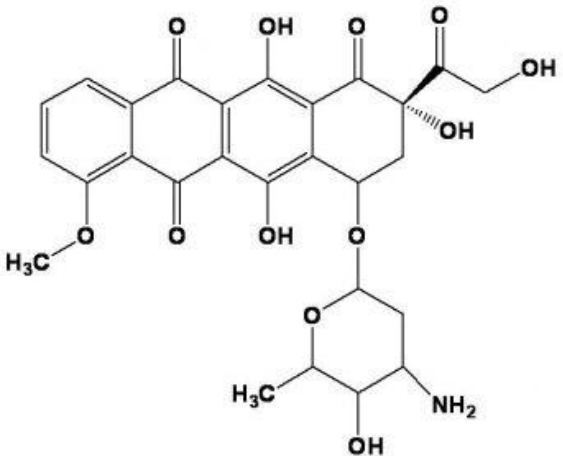

Figure 2.10 : Chemical structure of doxorubicin. (Retrieved from Inspiralis, 2006)

The anthracycline drug, Doxorubicin (or adriamycin), was first introduced by Arcamone *et al*. (1969) in the 1970's. Doxorubicin is the 14-hydroxy derivative of daunomycin extracted from *Streptomyces peucetius* var. *caesius*. It is commonly used in

the treatment of broad range of tumors, including breast, ovarian, bladder, gastric, thyroid cancer, srcoma, Wilms' tumorand neuroblastoma (Page and Takimoto, 2004).

There are different mechanisms have been proposed for the cytostatic and cytotoxic actions of doxorubicin. These include interference with macromolecular biosynthesis through DNA intercalation, free radicals generation, lipid peroxidation, formation of DNA adduct and DNA cross-linking, interference with DNA unwinding and DNA helicase action, direct membrane interaction, induction of DNA damage through inhibition of topoisomerase II and direct induction of apoptosis. However, the induction of DNA strand breaks by inhibition of nuclear enzyme topoisomerase II is suggested to be the primary mechanism of doxorubicin action (Gewirtz, 1999).

Chemoresistance was developed in bone cancer cells due to the overexpression of GST-P when the cells are exposed to doxorubicin. GST-P was claimed as the main cause of drug resistance due the suppression of GST-P in HOS cells was shown to increase apoptosis of the cells in response to doxorubicin. Indeed, GST-P-mediated activation of ERK 1/2 may be the mechanism of the development of drug resistance (Huang *et al*., 2007). Besides, Awasthi *et al*. (1996) able to enhance cytotoxicity of doxorubicin on human lung cancer cell line by enthacrynic acid inhibition on GSTs. Other than GST-P, Nielsen *et al*. (1996) proposed several other mechanisms of resistance developed in cell in response to anthracycline exposure. This includes P-glycoprotein-mediated multidrug resistance, Non P-glycoprotein-mediated multidrug resistance, vesicular drug compartmentalization, altered topoisomerase II activities and enhanced DNA repair.

The major limitation of clinical utilization of doxorubicin is cardiotoxicity in the aspect of cumulative dosage of the drug. Besides, the side effects of doxorubicin include

myelotoxicity, stomatitis, alopecia, diarrhea, dermatitis, red urine, anaphylactoid reaction, nausea and vomiting (Page and Takimoto, 2004).

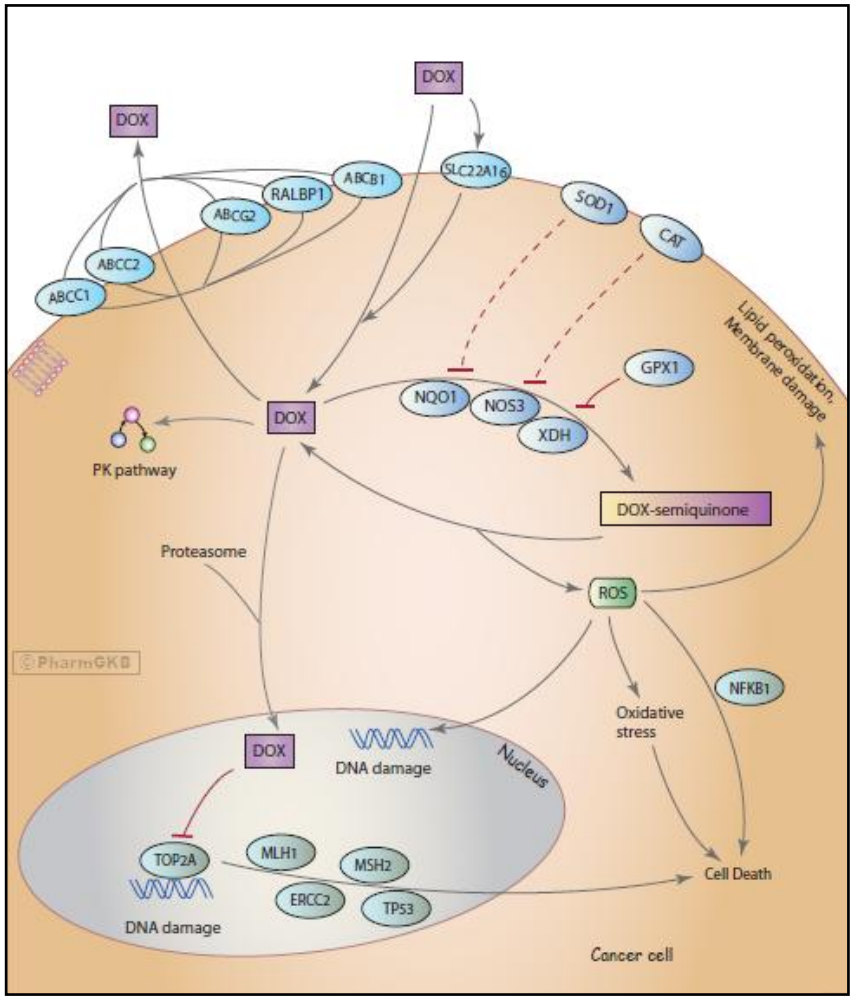

Figure 2.11 : Representation of the mechanisms involve in the action of doxorubicin (DOX) in cancer cell. Mechanisms described in the diagram include DNA intercalation, inhibition of topoisomerase II (TOP2A), generation of reactive oxygen species (ROS) which in turn lead to DNA damage, lipid peroxidation and membrane disruption, pharmacokinetics (PK) pathway and transporters associated with drug resistance. (Adapted from Thorn *et al*., 2011)

2.6 NATURAL PRODUCTS

Studies on natural products not only revealed new insight into fundamental biological mechanisms but also discovered new pharmaceutical or biotechnological possibilities of immediate use. Secondary metabolites which produced by different enzymatic pathways in plants to gain evolutionary advantages, has been reported to have a wide range of bioactivities. Besides, plant based biologically active cyclotides which have a unique protein folding, has been provides a framework in protein engineering and drug design applications (Bohlin *et al*., 2010).

There are numerous reports on natural products which have been proven to have significant biological effects against different types of cancer. Plant containing cardiac glycosides such as convallatoxin, olenandrin, proscillaridin A, digitoxin and digoxin, were identified to exhibit cytotoxic activity against colorectal cancer cell lines (HT-29, HCT-116 and CC20) with IC_{50} values ranging from 0.007-4.1 μM (Felth *et al.*, 2009). Curcumin, a yellow pigment derived from *Curcuma longa* Linn, was reported to induce apoptosis of HT-29 colorectal cells by inducing the p53 which involved in apoptosis signalling pathway and regulating the expression of apoptosis related proteins. Besides, it also suppressed carcinogenesis of skin, breast, stomach and liver (Song *et al.*, 2005). Naphthoquinones and their respective analogs isolated from *Avicennia* plants, phenylpropanoids and phytoquinoids isolated from *Illicium* plants, and phenylpropanoid of sucrose, vanicoside B and lapathoside A from *Polygonum lapathifolium*, have displayed remarkable anti-tumor-promoting activities against Eptein-Barr virus early antigen (EBV-EA) activation which induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cell line (Itoigawa *et al*., 2001; 2004; Takasaki *et al*., 2001). Finally, plant stress hormone, methyl jasmonate, exert selective cytotoxicity towards tumor cells by the induction of severe intracellular ATP depletion, induction of cellular re-differentation via mitogen-activated protein kinase (MAPK) pathway and induction of

apoptosis via hydrogen peroxide generation and Bcl-2 proapoptotic proteins (Cohen and Flescher, 2009).

2.7 PLANT POLYPEPTIDES

In recent decades, plant polypeptides have been extensively studied due to the presence of unique pharmacologically active polypeptides in several plant species (Gerlach *et al.*, 2010; Witherup *et al.*, 1994; Bokesch *et al.*, 2001), similarity with endogenous signaling compounds in animals which make use of peptides (Saether *et al.*, 1995), and the presence of transgenic plants that produced valuable recombinant polypeptides (Claeson *et al*., 1998; Yu *et al*., 2003).

Examples of established biologically active plant polypeptides are, the HIV-inhibitory macrocyclic polypeptides, palicourein from *Palicourea condensata* (Bokesch *et al.*, 2001), circulins A-F from *Chassalia parvifolia* (Gustafson *et al.*, 1994; 2000); cyclopsychotride A from *Psychotria longipes*, which inhibits binding of neurotensin to HT-29 cell membranes and also elevated intracellular Ca^{2+} concentration in cell lines without neurotensin receptors (Witherup *et al.*, 1994); kalata B1 peptide from *Oldenlandia affinis*, which has uterotonic activity (Saether *et al.*, 1995); finotin from *Clitoria ternatea* that possess antimicrobial and antifungal activities (Kelemu *et al.*, 2004); kalata B1 and B6 of *Oldenlandia affinis* and cycloviolacin O14 of *Viola odorata* that proved to have antihelmintic activity (Colgrave *et al.*, 2009); psyles A, C and E of *Psychotria leptothyrsa* which showed to have cytotoxic effect on lymphoma cell line U937-GTB (Gerlach *et al*., 2010); and the varv A and varv F of *Viola arvensis* and cycloviolacin O2 of *Viola odorata* which exhibited high cytotoxic activities on several cancer cell lines (Lindholm *et al.*, 2002). In addition, the four macrocycliccystine-knot peptides, kalata, circulin A and B, and cyclopsychotride, have been shown to have

antimicrobial, antifungal and haemolytic properties (Tam *et al*., 1999). Occurrence of highly stable small cyclic peptides in plants is common especially for those worked as antibiotics of microbial origin. Immunosuppressant, cyclosporine, is the best example to exemplified the pharmaceutical uses of these type of cyclic peptides (Craik *et al*., 1999).

According to Craik *et al.* (2001), cystine knot motif has been found in wide variety of peptides and proteins. Presence of cystine knot engendered a particularly high degree of chemical and biological stability to the protein molecule itself. So, the ability to synthesize and figure out the structures of the cystine knot containing peptides somehow offered a valuable framework in protein engineering (Gran *et al.*, 2000).

Thus, wide range of bioactivities, high stability of cytine knot motif and distinctive structure scaffold of peptide toxins like cyclotides (Gerlach *et al.*, 2010), can be harnessed for drugs design applications.

2.8 RESEARCH STATEMENT

Since GST-P has significance in cancer diagnosis and its over-expression contributes to chemoresistance in many cancers and become a major reason of chemotherapy failure and disease recurrence, finding of agents that can inhibit GST-P can be consider as an adjuvant in cancer treatment.

There was a number of potential inhibitors of GST-P has been found and designed. Some of the inhibitors were reported to have the ability to enhance cytotoxicity effects of clinically used anti-cancer drugs. However, some of the GST-P inhibitors are found have side effects and thus limited its clinical application.

In my research, HT-29 cell line is selected for the cytotoxicity assays and extraction of GST-P enzymes as the cell line expresses high level of GST-P. In fact,

HT-29 cell line was reported to be more resistant to cytotoxic agent compared to other cell lines.

Besides, plant-based GST-P inhibitor is targeted in my research as there are numerous reports on natural products which have been proven to have significant biological effects against different types of cancer.

The significance of this research would be the finding of GST-P inhibitors in local plant that can be used as a medication drug to overcome anti-cancer drug resistance during cancer treatment.

2.9 OBJECTIVES

The objectives of this study are listed out as below:

- To screen plant extracts with inhibitory activity toward GST-P.
- To evaluate the behavior of inhibitors toward GST-P.
- To evaluate the ability of inhibitors to potentiate cytotoxicity of selected drugs on HT-29 cell line.

3.0 MATERIALS & METHODS

3.1 MATERIALS

3.1.1 PLANT MATERIALS

31 local plants (43 samples) selected in this study were collected and prepared by Mr. Ghazali and Mr. Izuwan from University of Malaya. Plant samples were authenticated by plant taxonomist, Prof. Dr. Ong Hean Chooi. All of the samples were given in dried form and readily separated into different plant parts. The plant parts consist of leaf, fruit, flower, branches and also the whole plant. Plant samples used in this study included *Anacardium occidentale* (branch, fruit & flower), *Andrographis paniculata* (branch, leaf), *Artocarpus heterophyllus* (leaf), *Averrhoa bilimbi* (branch, leaf), *Cinnamomum zeylanicum* (branch), *Commelina nudiflora* (whole plant), *Euodia redlevi* (flower & fruit), *Eupatorium odoratum* (branch), *Fagraea fragrans* (leaf & branch, fruit & flower), *Ficus auriculata* (leaf), *Garcinia atroviridis* (branch), *Garcinia mangostana* (branch, leaf), *Hibiscus tiliaceus* (leaf), *Ipomoea aquatica* (leaf), *Justicia gendarussa* (branch), *Lagerstroemia speciosa* (fruit, leaf), *Lawsonia inermis* (branch), *Leptospermum flavescens* (leaf, branch), *Macaranga conifera* (branch), *Melaleuca cajuputi* (fruit & flower), *Moringa oleifera* (branch), *Orthosiphon stamineus* (leaf), *Oxalis barrelieri* (root), *Peltophorum pterocarpum* (fruit, leaf), *Pereskia bleo* (fruit), *Piper nigrum* (leaf, branch, fruit), *Scoparia dulcis* (root), *Strobilanthes crispa* (branch), *Tetracera indica* (fruit), *Vitex* sp. (branch, leaf) and *Vitex trifolia 'purpurea'* (branch, leaf). Table 7.1 in Appendix I indicates the details of origin and references of plant parts.

3.1.2 CELL MATERIALS

The human colon adenocarcinoma HT-29 cell line and fetal lung fibroblast MRC-5 cell line were purchased from American Type Culture Collection (ATCC, USA).

3.1.3 CHEMICAL REAGENTS AND DRUGS

All of the chemical reagents used in this study were of analytical grade unless stated. The chemicals and drugs used included:

0.5M Tris-HCl buffer, pH 6.8 (Bio-Rad), 1.5M Tris-HCl buffer, pH 8.8 (Bio-Rad), 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-aldrich), 2-Mercaptoethanol (Merck), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-aldrich), 30% Acrylamide/Bis solution, 37.5:1 (2.6% C) (Bio-Rad), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Fluka), Accutase® solution (Sigma-aldrich), Acetonitrile (ACN) (J. T. Baker), Ammonium bicarbonate ($(NH_4)HCO_3$) (Sigma-aldrich), Ammonium persulfate (APS) (Bio-Rad), Ammonium sulfate ($(NH_4)_2SO_4$) (Systerm®), Amphotericin B (PAA), Brilliant blue G-250 (Sigma-aldrich), Bromophenol blue (Sigma-aldrich), CelLytic™ M (Mammalian cell lysis/extraction reagent) (Sigma-aldrich), cis-Diamineplatinum(II) dichloride (Cisplatin) (Sigma-aldrich), Dichloromethane (DCM) (Systerm®), Dimethyl sulfoxide (DMSO) (Sigma-aldrich), Doxorubicin hydrochloride (Sigma-aldrich), Eagle Minimum Essential Medium, with L-glutamine, powder (EMEM) (Sigma-aldrich), Ethacrynic acid (Sigma-aldrich), Ethanol (Systerm®), Foetal bovine serum (FBS) (PAA), Formaldehyde (Systerm®), Glacial acetic acid (Systerm®), Glycerol (Systerm®), Glycine (Systerm®), Hydrochloric acid (HCl) (Systerm®), IEF anode buffer (50X) (Invitrogen), IEF cathode buffer, pH 3-10 (10X) (Invitrogen), IEF sample buffer, pH 3-10 (2X) (Invitrogen), L-Glutathione reduced (GSH) (Sigma-aldrich), Mark 12™ unstained standard (Invitrogen), Mass standards kit for the 4700 proteomics analyzer (includes calibration mixture 1, 4700 proteomic analyzer calibration mixture, digested β-galactosidase, α-cyano-4-hydroxycinnamic acid (CHCA)) (Applied Biosystems), Methanol (Systerm®), N,N,N',N'- Tetramethylethylenediamine (TEMED) (Bio-Rad), Ortho-phosphoric acid (H_3PO_4) (Systerm®), Penicillin/Streptomycin (PAA), Phosphate buffered saline powder,

pH 7.4 (PBS) (Sigma-aldrich), Protease inhibitor cocktail (Sigma-aldrich), RPMI-1640 Medium (with L-glutamine, without sodium bicarbonate, powder) (Sigma-aldrich), SERVA IEF protein markers 3-10 (Invitrogen), Silver nitrate ($AgNO_3$) (Systerm®), Sodium bicarbonate ($NaHCO_3$) (BDH), Sodium carbonate (Na_2CO_3) (R&M Chemicals), Sodium dihydrogen phosphate (NaH_2PO_4) (Systerm®), Sodium dodecyl sulfate (SDS) (Sigma-aldrich), Sodium hydroxide (NaOH) (Systerm®), Sodium pyruvate (Sigma-aldrich), Sodium thiosulfate ($Na_2S_2O_3$) (Systerm®), Trichloroacetic acid (TCA) (R&M Chemicals), Trifluoroacetic acid (TFA) (Fisher Scientific), Tris base (Promega), Trypan blue (Sigma-aldrich), Trypsin (Sigma-aldrich), Trypsin-EDTA (PAA).

3.1.4 EQUIPMENTS

- -20 ℃ refrigerator
- 4 ℃ refrigerator
- -80 ℃ refrigerator
- ÄKTA Prime Plus
- ÄKTA Purifier FPLC
- Autoclave machine
- Centrifuge machine (refrigerated and non-refrigerated)
- CO_2 incubator
- Cold room
- Freeze-dryer
- Fraction collector
- Fume hood
- GSTrap HP column, 1 mL
- Haemocytometer

- Hot plate and magnetic stirrer
- Hot water incubator
- Ice maker
- Image scanner
- Lamina hood
- Liquid nitrogen tank
- MALDI TOF mass spectrometer
- Microplate mixer
- Microplate reader
- Microscope
- Microsep™ centrifugal concentrator
- Mini-PROTEAN® tetra cell
- Orbital shaker
- Oven
- pH meter
- PowerPac Basic™ power supply
- Rotary evaporator
- SpeedVac concentrator
- Ultrapure water system
- Ultrasonic cleaner
- UV-Vis spectrophotometer
- Vortex mixer
- Water distiller
- Weighing machine
- XCell *SureLock*® Mini-Cell

OVERVIEW OF METHODOLOGY

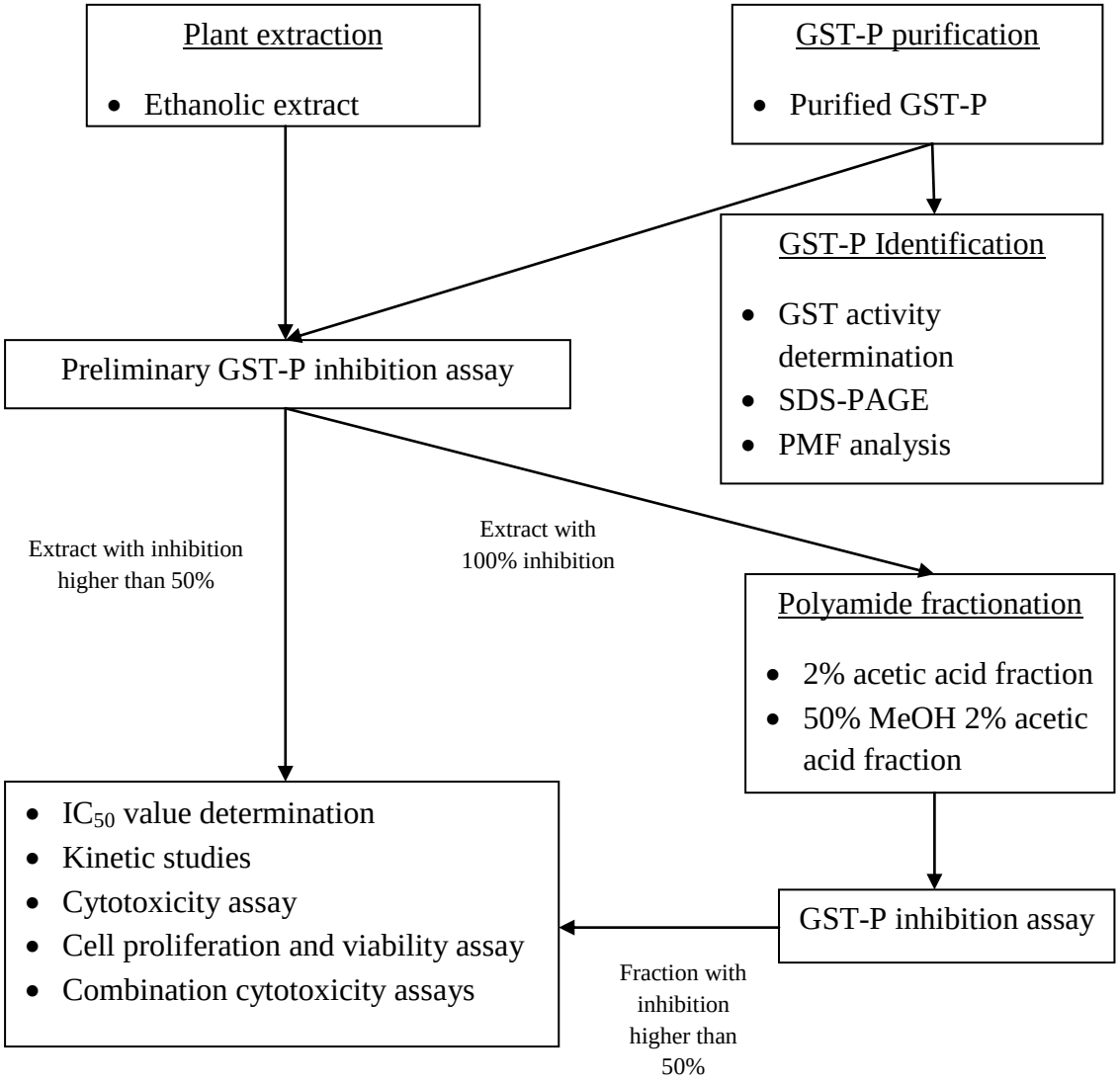

Figure 3.1 : Overview of methodology.

3.2 METHODS

3.2.1 PLANT EXTRACT PREPARATION

The plant samples (a total of 43 samples) supplied were readily ground into small pieces. The extraction was done according to Claeson *et al.* (1998), with some modification. In brief, 20 g of each plant sample was weighed and soaked in 200 mL of Dichloromethane (DCM). The mixture was shaken overnight at 100 rpm on orbital shaker in cold room. Then, the mixture was filtered with filter paper and the plant residue was then dried in fume hood. After that, dried residue was soaked in ethanol: water at a ratio of 1:1 (100 mL ethanol: 100 mL water). The mixture was then shaken overnight at 100 rpm on orbital shaker in cold room. Then, the mixture was filtered and the filtrate was collected. Filtrate was washed with DCM using separating funnel and then sent for rotary evaporation at 36-38 ℃. The concentrated extract will be denoted as ethanolic extract and it was transferred into a universal bottle and frozen at -80 ℃ before subjected to freeze-drying. Freeze-dried ethanolic extract is then stored at -20 ℃ prior to use.

3.2.2 HT-29 CELL CULTURE AND LYSIS

3.2.2.1 PREPARATION OF MEDIA AND SOLUTIONS

The media and solutions used in this experiment were prepared as stated in Appendix II.

3.2.2.2 CELL REVIVAL

A vial of HT-29 cell line was removed from liquid nitrogen and quick thawed in a water bath at 37 ℃. The cells were then transferred into a centrifuge tube containing 1 mL of 20% supplemented RPMI 1640 medium. The cells were spun at 1000 rpm for 5 minutes. Then, the supernatant was discarded and 1 mL of 20% supplemented RPMI 1640 medium was added and mixed. The cells were then transferred into a 25 cm^2 culture flask (Nunc, Denmark) which contained 4 mL of 20% supplemented RPMI 1640 medium. The cells were cultured in humidified 5% CO_2 incubator at 37 ℃.

3.2.2.3 CELL MAINTENANCE

After the HT-29 cells adherent and started to grow, the medium was discarded. The cells were rinsed with PBS and 5 mL of 10% supplemented RPMI 1640 medium was then added to maintain the growth of the cells. Medium was changed regularly until the cells confluent.

3.2.2.4 CELL SUBCULTURATION

When the monolayer HT-29 cells were confluent, the cells were then trypsinized with trypsin-EDTA and PBS at the ratio of 1: 3 (1 mL of trypsin-EDTA: 3 mL of PBS). The medium was removed and the monolayer cells were rinsed with PBS prior to trypsinization. In the process of trypsinization, the cells were incubated in 5% CO_2 incubator at 37 ℃ for 5-10 minutes, until the cells started to detach (this was assisted by tapping the sides of the flask for a few times). The cells were then transferred into a centrifuge tube which contained 1 mL of 10% supplemented RPMI 1640 medium, and then spun at 1000 rpm for 5 minutes. After that, the supernatant was discarded and the

cell pellet was re-suspended in 3 mL of 10% supplemented RPMI 1640 medium. 1 mL of cell suspension was transferred into each culture flask (a total of 3 flasks) and maintained with 4 mL of 10% supplemented RPMI 1640 medium.

3.2.2.5 CELL CRYOPRESERVATION

In order to maintain the availability of cell line, cell line stocks were preserved and stored in liquid nitrogen. Confluent monolayer HT-29 cells were trypsinized with trypsin-EDTA and PBS at the ratio of 1: 3 (1 mL of trypsin: 3 mL of PBS) in 5% CO_2 incubator at 37℃ for 5-10 minutes, after removed the medium and rinsed the monolayer with PBS. The cell suspension was then transferred into a centrifuge tube with 1 mL of 10% supplemented RPMI 1640 medium. The cells were spun at 1000 rpm for 5 minutes. Supernatant was then removed and the cells were re-suspended in freezing medium and transferred into cryopreservation vials. The vials were placed in vapour phase of liquid nitrogen for 3-4 h before transferred to cryocane in liquid nitrogen (-196℃) for long term storage. The liquid nitrogen tank was stored in cold room at 4℃.

3.2.2.6 CELL LYSIS

Medium of a flask of confluent monolayer HT-29 cells was removed and the cells were rinsed with PBS and then trypsinized with trypsin-EDTA and PBS at a ratio of 1: 3 (1 mL of trypsin EDTA: 3 mL of PBS) in 5% CO_2 incubator at 37℃ for 5-10 minutes. Cells suspension was then transferred into a centrifuge tubes which contained 1 mL of PBS. The cells were then spun at 1000 rpm for 5 minutes. After that, the supernatant was discarded and the cells were re-suspended in 1 mL of PBS. In order to

determine the number of cells in the cell suspension, cell counting was done with haemocytometer. 100 µL of cells were mixed with 900 µL of 0.4% trypan blue (0.2 g of trypan blue dissolved in 50 mL of distilled water) and 20 µL was pipette and loaded onto loading port of the haemocytometer. The haemocytometer was then observed under the microscope for cell counting. There were nine 1 mm x 1 mm boxes observed on the counting area. Only cells scattered in the four cornered boxes and the center box were counted. Number of cells counted was used to determine the amount of CelLytic™ M to be added in for cell lysis. The cell concentration per ml was calculated using the following formula:

$C_1 = N \times D \times 1/5 \times 10^4$

Where,

C_1 = initial cell concentration per ml

N = total cell count for the 5 boxes

D = correction for the tryphan blue dilution

1/5 = correction to give mean cells per box

10^4 = conversion factor for counting chamber

The remaining cells were then spun at 500 xg for 5 minutes. Supernatant was then discarded and cells were re-suspended in 250-350 µL of CelLytic™ M (depends on the number of cells counted) with protease inhibitor cocktail at the ratio of 10: 1. The mixture was shook for 15 minutes and then spun at 15,000 xg for another 15 minutes at 4 ℃. The clear supernatant (lysate) was collected and stored at -80 ℃ for further use.

3.2.3 GLUTATHIONE S-TRANSFERASE π PURIFICATION

3.2.3.1 PREPARATION OF BUFFERS AND SOLUTIONS

The buffers and solutions used in this experiment were prepared as stated in Appendix II.

3.2.3.2 AFFINITY CHROMATOGRAPHY

GSTrap HP affinity column (1 mL) was used to purify glutathione S-transferase π from the lysate of HT-29 cell line. The GSTrap HP column was pre-packed with GSH-Agarose matrix for one-step purification of glutathione S-transferase (GST) tagged protein. The GSTrap HP column was connected to ÄKTA Prime Plus which used to monitor the purification process. Flow rate was set at 0.3 mL/min. The system was first equilibrated with 25 mM sodium phosphate buffer. After that, 1 mL of lysate was loaded into the system. The lysate was eluted through the column with 25 mM sodium phosphate buffer, pH 7.4. All the unbound proteins were eluted out from the column once it entered the column. To elute the bound GST protein, eluting buffer was changed to 10 mM reduced glutathione in 25 mM sodium phosphate buffer, pH 7.4. Fractions were collected once the peak observed and it was then subjected to GST activity assay. Fractions (GST eluent) with high activities were pooled together and concentrated with 1K Microsep™ centrifugal concentrator, spun at 6000 xg for 90 minutes at 4℃. Concentrated GST was subjected to SDS-PAGE analysis and IEF electrophoresis, the remaining kept at -80℃ before subjected to freeze-drying. Lyophilized GST was kept at -20℃ for further use.

3.2.4 GLUTATHIONE S-TRANSFERASE π IDENTIFICATION

3.2.4.1 GLUTATHIONE S-TRANSFERASE (GST) ACTIVITY DETERMINATION

3.2.4.1.1 PREPARATION OF BUFFERS AND SOLUTIONS

The buffers and solutions used in this experiment were prepared as stated in Appendix II.

3.2.4.1.2 GLUTATHIONE S-TRANSFERASE (GST) ACTIVITY ASSAY

The activity of the glutathione S-transferase was determined according to the spectrophotometric enzyme assay described by Habig *et al.* (1974). The condition of the GST activity assay as below:

Blank: 3.00 mL of buffer A

Control: 2.90 mL of buffer A + 0.05 mL of 60 mM reduced glutathione + 0.05 mL of 60 mM CDNB

Sample: 2.85 mL of buffer A + 0.05 mL of 60 mM reduced glutathione + 0.05 mL of GST solution + 0.05 mL of 60 mM CDNB

Changes of absorbance at the wavelength of 340 nm were recorded for 10 minutes at 25℃, using Jasco V630 UV-Vis spectrophotometer. The enzyme activity and specific activity of GST were then determined by using the formula below. The molar extinction coefficient of CDNB is 9600 $M^{-1}cm^{-1}$.

Enzyme activity, μmol/min/mL = $\frac{\Delta A}{t} \times \frac{1}{kl} \times \frac{Vt}{Vs}$

Where,

ΔA = absorbance changes

t = time (min)

k = molar extinction coefficient ($M^{-1}cm^{-1}$)

l = diameter of cuvette (cm)

Vt = total volume of assay (mL)

Vs = volume of sample (mL)

$$\text{Specific activity, μmol/min/mg protein} = \frac{total\ enzyme\ activity\ (\mu mol\ /min)}{total\ protein\ content\ (mg)}$$

3.2.4.2 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The concentrated GST eluent obtained from affinity chromatography (Section 3.2.3.2) was subjected to SDS-PAGE analysis by using a 4% stacking: 12% resolving gel. This was carried out according to Laemmli (1970), using Mini-PROTEAN® Tetra Cell (Bio-rad).

3.2.4.2.1 PREPARATION OF BUFFERS AND SOLUTIONS

The buffers and solutions used in this experiment were prepared as stated in Appendix II.

3.2.4.2.2 GEL PREPARATION

4% stacking: 12% resolving gel monomer solutions were prepared as shown in Appendix II. The monomer solutions were prepared by mixing all reagents except for TEMED and 10% APS. The solutions were then degassed for 15 minutes. After that, TEMED and 10% APS were added and the mixtures were swirled gently to initiate polymerization prior to be loaded into the casting cassette. The solutions were then load into the casting cassette in the order of resolving monomer solution first, followed by stacking monomer solution after the resolving gel completely polymerized. Distilled water was used to overlay the resolving monomer solution. A comb was inserted into the stacking monomer solution to form well. The 4% stacking: 12% resolving gel was leave overnight in order to achieve complete polymerization before sample loading.

3.2.4.2.3 SAMPLE LOADING

Before loading the sample (concentrated GST eluent), sample was mixed with SDS sample buffer at the ratio of 1: 2 (10 µL of sample: 20 µL of SDS sample buffer). 50 µL of 2-mercaptoethanol was added to 950 µL of SDS sample buffer prior to use. Sample buffer mixed sample was then heated at 95℃ for 4 minutes to denature the protein sample. Then, 10 µL of sample buffer mixed sample was loaded into one the well of the gel. 4 µL of Mark 12™ unstained standard, which used as the protein marker, was loaded into another well.

3.2.4.2.4 ELECTROPHORESIS

Electrophoresis was carried out at 150 V for approximately 1 hour. The process was stopped when the tracking dye reached about 1 cm from the bottom of the gel. The gel was then removed and subjected to colloidal coomassie blue staining.

3.2.4.3 COLLOIDAL COOMASSIE BLUE STAINING

3.2.4.3.1 PREPARATION OF COLLOIDAL COOMASSIE BLUE SOLUTION

The colloidal coomassie blue solution was prepared as shown in Appendix II.

3.2.4.3.2 GEL STAINING

The colloidal coomassie blue staining was done according to Neuhoff *et al*. (1988). Prior to staining, the colloidal coomassie blue solution was mixed with methanol at the ratio of 4: 1 (80 mL of colloidal brilliant blue: 20 mL of methanol). The gel removed from SDS-PAGE was then stained by immersed the gel in the colloidal solution and leave overnight on orbital shaker. After that, the gel was de-stained with 20% methanol (20 mL of methanol: 80 mL of distilled water).

3.2.4.4 MOLECULAR WEIGHT DETERMINATION

Single protein band appeared on the lane of sample (GST eluent) was compared with the bands of standard marker. Relative mobility, R_f, of each band was determined by dividing the distance migrated by the band with the distance migrated by the tracking

dye. A standard curve of log molecular weight against R_f of standard marker was constructed. The molecular weight of the single protein band can then be determined from the curve.

3.2.4.5 PEPTIDE MASS FINGERPRINT (PMF) ANALYSIS

After determined the molecular weight of the sample protein band, the protein band was then excised for PMF analysis at Medical Biotechnology Lab, Faculty of Medicine, University of Malaya, using MALDI-TOF mass spectrometer (ABI 4800 Plus).

3.2.4.5.1 PREPARATION OF SOLUTIONS

The solutions used in this experiment were prepared as stated in Appendix II.

3.2.4.5.2 DESTAINING AND TRYPSIN DIGESTION

This method was done according to Speicher *et al.* (2000) with some modification. After excised the protein band from stained SDS-PAGE gel, the gel slice was de-stained with 200 µL of 200 mM $(NH_4)HCO_3$ in 50% ACN for 45 minutes at 37℃. The supernatant was then discarded. Destaining process was repeated until the stain no longer visible. Gel slice was then dried using SpeedVac concentrator. Dried gel slice was then re-hydrated with 20 µL of trypsin solution for an hour. Then, an additional 50 µL of 40 mM $(NH_4)HCO_3$ in 10% ACN was added and incubation was continued for 16-18 h at 37℃. After the incubation, supernatant was removed into a cleaned tube (extract 1). Then, 50 µL of 0.1% TFA was added on the gel and incubated

for 45 minutes at 37℃. The supernatant was then removed and pooled into the tube with extract 1.

3.2.4.5.3 PMF ANALYSIS

The extract 1 was mixed with CHCA matrix solution at a ratio of 1: 1 (1 µL of extract 1: 1 µL of CHCA matrix solution) before spotted on the sample plate. 0.5 µL of diluted CHCA matrix was spotted on one of the spot on the sample plate, followed by 0.5 µL of CHCA mixed extract 1 on the same spot. Digested β-galactosidase was used as the control for PMF analysis. The sample was then allowed to air evaporated before subjected to MALDI-TOF (ABI 4800 PLUS) at Medical Biotechnology Lab, Faculty of Medicine, University of Malaya.

3.2.4.6 ISOELECTRIC FOCUSING (IEF) ELECTROPHORESIS

To determine the number of pi isoform of GST exist in the sample, IEF was done. IEF electrophoresis separated proteins according to their isoelectric point (pI). The electrophoresis was done according to manufacturer's manual on pre-cast 12-welled Novex® pH 3-10 IEF mini gel using XCell *SureLock*® Mini-Cell (Invitrogen).

3.2.4.6.1 BUFFERS PREPARATION

The buffers used in this experiment were prepared as stated in Appendix II.

3.2.4.6.2 SAMPLE AND BUFFER LOADING

Prior to loading of sample, 5 μL of GST eluent was mixed with 5 μL of 2X IEF sample buffer pH 3-10. A total of 10 μL sample was loaded on the gel, with 2 μL of SERVA IEF protein markers 3-10 loaded on another lane which to be used as reference. The upper buffer chamber of XCell *SureLock*® Mini-Cell is loaded with 200 mL of IEF cathode buffer and the lower buffer chamber is loaded with 600 mL of IEF anode buffer.

3.2.4.6.3 ELECTROPHORESIS RUNNING CONDITION

The electrophoresis was started with 100 V at 7 mA and kept constant for 1 hour, followed by 200 V for another 1 hour. Lastly ran at 500 V for 30 minutes. The final current reading is expected to be 5 mA.

3.2.4.7 VORUM SILVER STAINING

Other than Coomassie blue and fluorescent dyes, silver staining is also one of the common methods available to visualize proteins separated by gel electrophoresis. The Vorum silver staining procedures adopted from Mortz *et al*. (2001) are compatible with mass spectrometry.

3.2.4.7.1 PREPARATION OF SOLUTIONS

The buffers used in this experiment were prepared as stated in Appendix II.

3.2.4.7.2 STAINING PROCEDURES

After the electrophoresis, gel was removed from the cassette and placed into a tray containing 12% trichloroacetic acid and soaked for 30 minutes. After that, the solution was discarded and gel was soaked in fixing solution for approximately 2 h. After discard the fixing solution, the gel was washed 3 times with washing solution, each time took 20 minutes. After the washing step, the gel was sensitized with sensitizing solution for 2 minutes. Then, sensitizing solution was discarded and the gel was washed thrice, one minutes each, with de-ionized water. It is then followed by addition of silver staining solution and incubated for 20 minutes. After completed the staining step, staining solution was poured off and the gel was rinsed twice, each time 10 seconds, with de-ionized water. Protein image of the gel was developed by incubating gel in developing solution for 3-5 minutes. The reaction was stopped as soon as the desired intensity of protein bands observed. The reduction reaction was stopped by the addition of terminating solution and incubated of 5 minutes. The whole staining process was carried out with gentle rotation. The gel is then preserved in preservation solution. Image of the gel was scanned with ImageScanner III (GE Healthcare).

3.2.5 PRELIMINARY GST-P INHIBITION ASSAY

Ethanolic extracts of the 43 plant samples were tested in this experiment. 10 mg/mL of GST-P solution was prepared by dissolving 10 mg of freeze-dried GST-P in 1 mL of buffer A and 10 mg/mL of the ethanolic extract was prepared by dissolving 10 mg of freeze-dried ethanolic extract in 1 mL of buffer A. Condition of the GST-P inhibition assay as below:

Blank: 3.00 mL of buffer A

Control: 2.90 mL of buffer A + 0.05 mL of 60 mM reduced glutathione + 0.05 mL of 60 mM CDNB

GST-P only: 2.85 mL of buffer A + 0.05 mL of 60 mM reduced glutathione + 0.05 mL of 10 mg/mL of GST-P solution + 0.05 mL of 60 mM CDNB

GST-P with ethanolic extract: 2.85 mL of buffer A + 0.05 mL of 60 mM reduced glutathione + 0.05 mL of 10 mg/mL of GST-P solution + 0.05 mL of 10 mg/mL of ethanolic extract + 0.05 mL of 60 mM CDNB

Changes of absorbance at the wavelength of 340 nm were recorded for 10 minutes at 25 ℃, using Jasco V630 UV-Vis spectrophotometer. The specific activity of GST-P (SA_{GST-P}) was then calculated (formula used same as the formula stated in Section 3.2.4.1.2) and the relative change in SA_{GST-P} was then determined as below. The molar extinction coefficient of CDNB is 9600 $M^{-1}cm^{-1}$.

$$\text{Relative reduction in } SA_{GST-P} = \frac{SA_{GST-P} - SA_{GST-P \text{ with ethanolic extract}}}{SA_{GST-P}} \times 100\%$$

3.2.6 BIOASSAY-GUIDED FRACTIONATION

3.2.6.1 PREPARATION OF SOLUTIONS

The solutions used in this experiment were prepared as stated in Appendix II.

3.2.6.2 POLYAMIDE FILTRATION AND FRACTIONATION

Based on the results of previous experiment (Section 3.2.5), selected ethanolic extracts with 100% inhibition activity on GST-P were further fractionated with polyamide column chromatography. The lyophilized ethanolic extract was re-dissolved in 2% acetic acid and filtered. Polyamide resin (Fluka, Germany) was pre-swollen in 2% acetic acid and packed in a 25 mL glass column. Initially, Polyamide column was equilibrated with 50% methanol 2% acetic acid and 2% acetic acid before application of ethanolic extract onto the column. After the injection of ethanolic extract into the system, the unbound sample was eluted with 2% acetic acid, followed by 50% methanol 2% acetic acid for the compounds slightly bound to the resin. Eluates were then rotary evaporated and freeze-dried prior to further uses. The chromatography was monitored with ÄKTA Purifier FPLC (GE Healthcare, UK) equipped with fraction collector.

Fractions were then subjected for GST-P inhibition assay (refer to Section 3.2.5).

3.2.7 IC_{50} VALUE DETERMINATION WITH GST ACTIVITY ASSAYS

Plant ethanolic extracts and fraction with GST-P inhibition activity higher than 50% (refer to Section 4.4) were selected for this experiment.

Basically, the GST-P inhibition assays were done on 96-well microplate (Nunc, Denmark). Plant samples (ethanolic extract/fraction) prepared at different concentrations were mixed with reaction mixture containing 0.2 mg/mL of GST-P and 1 mM of reduced glutathione (GSH) in buffer A. Reactions were initiated with the addition of 1 mM of CDNB and changes in absorbance were recorded at the wavelength of 400 nm (with reference wavelength at 500 nm) for 10 minutes at 25℃, using Sunrise™ Absorbance Reader (Tecan). The assay condition includes:

Blank: 250 μL of buffer A

Control 1: 230 μL of buffer A + 10 μL of 25 mM GSH + 10 μL of 25 mM CDNB

GST-P only: 220 μL of buffer A + 10 μL of 25 mM GSH + 10 μL of 25 mM CDNB + 10 μL of 5 mg/mL GST-P

GST-P and plant sample: 220 μL of buffer A + 10 μL of 25 mM GSH + 10 μL of 25 mM CDNB + 10 μL of 5 mg/mL GST-P + 10 μL of plant sample

Control 2: 240 μL of buffer A + 10 μL of plant sample

The percentage inhibition on GST-P activity (%) by each sample was calculated as following:

$$\text{Percentage of inhibition, \%} = \{[(A_{\text{GST-P}} - A_{\text{control 1}}) - (A_{\text{GST-P and plant sample}} - A_{\text{control 1}} - A_{\text{control 2}})] / (A_{\text{GST-P}} - A_{\text{control 1}})\} \times 100\%$$

Where, $A_{\text{control 1}}$, $A_{\text{control 2}}$, $A_{\text{GST-P}}$ and $A_{\text{GST-P and plant sample}}$ were the absorbance at 400 nm (with reference wavelength of 500 nm) for Control 1, Control 2, GST-P and GST-P and plant sample, respectively.

The GST-P inhibition ability of plant ethanolic extracts and fraction were expressed as IC_{50} value, which indicates the inhibition concentration at which 50% of GST-P activities were inhibited. The IC_{50} value was determined from the graph of percentage of inhibition on GST activity (%) versus concentration of plant sample, generated using the software Microsoft Excel.

3.2.8 KINETIC STUDIES

Rate of reaction was measured as described by van Haaften *et al.* (2003). In order to determine the inhibitory mechanism of plant samples (ethanolic extract/fraction) on GST-P activity, the substrate (either CDNB or GSH) concentrations were varied. In this study, the CDNB concentration was maintained at 1 mM while the concentration of GSH varied in the reaction mixture of buffer A. The concentration of plant samples was kept constant at the IC_{50} value which determined from previous experiment (Section 3.2.7) and the concentration of GST-P maintained at 0.2 mg/mL in 1 mL reaction mixture. Changes in GST-P activity were monitored at the wavelength of 340 nm at 25 ℃ for 10 minutes using Jasco V630 UV-Vis spectrophotometer. Spontaneous reaction between GSH and CDNB in the absence of GST-P was corrected from the reading obtained. V_{max} and K_m values of GST-P with or without addition of plant samples were generated using SigmaPlot 12.0 graph and analysis software.

3.2.9 CYTOTOXICITY ASSAY

3.2.9.1 PREPARATION OF DILUENTS AND SOLUTIONS

The diluents and solutions used in this experiment were prepared as stated in Appendix II.

3.2.9.2 CELL CULTURE

In order to examine the toxicity effects of selected plant samples (ethanolic extract/fraction) against normal human cells, cytotoxicity of the plant samples on fetal lung fibroblast MRC-5 cell line were tested.

The MRC-5 cells used in this experiment were cultured in 10% supplemented EMEM (diluent X). Cell culture was maintained in humidified 5% CO_2 incubator at 37 ℃. The cells were grown to 90-100% confluence before proceed to seeding.

3.2.9.3 CELL PREPARATION AND SEEDING

Cytotoxicity effect of plant samples (ethanolic extract/fraction), doxorubicin hydrochloride, cisplatin and ethacrynic acid against MRC-5 cells were tested. Monolayer MRC-5 cells with 90-100% confluence were detached using accutase solution and transferred into a centrifuge tube containing 1 mL of diluent X. The cells were spun at 1000 rpm for 5 minutes. Then, the supernatant was discarded. 1 mL of diluent X was added and the cells were gently resuspended.

10 µL of cell suspension was mixed with 90 µL of trypan blue solution in a microcentrifuge tube and then 10 µL of the mixture was transferred to a haemocytometer for cell counting. Percentage of cell viability was determined and viability should be at least 95% in order to proceed with the assay.

Cells were seeded in flat bottom 96-well microplate (Nunc, Denmark) at a concentration of 2 x 10^5 cells/mL, in 100 µL culture medium per well. Cells were allowed to attach for 24 h in humidified 5% CO_2 incubator at 37 ℃.

3.2.9.4 SAMPLE DILUTION PREPARATION

Sample stock and sub-stock were prepared before conducting serial dilution. Sample stock was prepared by dissolving 0.02 g of plant sample (ethanolic extract/fraction) in 0.2 mL of 100% DMSO, which final concentration is 100 mg/mL.

Meanwhile, to prepare 1 mL of sub-stock at a concentration of 200 μg/mL containing 0.2% DMSO, 2 μL of stock was added to 998 μL of diluent X. Calculation can be done with the formula:

$M_1V_1 = M_2V_2$

Where,

M_1 = initial concentration

V_1 = volume of M_1 required

M_2 = final concentration

V_2 = final volume

After preparation of sub-stock, serial dilution was conducted using flat bottom 96-well microplate and multi-channel pipettor (Eppendorf, Germany). First of all, 150 μL of diluent Y was added to wells in row B to H (Appendix III, Figure 7.1). Then, 300 μL of sub-stock was added to wells in row A, at which the concentration of sample was 200 μg/mL. After that, 150 μL of solution in wells of row A was transferred to wells in row B, which create a second concentration at row B, which is 100 μg/mL. The dilution was continued until row G and 150 μL of solution in wells of row G was discarded. Hence, the volume in each well was 150 μL containing 0.2% DMSO. This is then followed by addition of 150 μL of diluent Y into every single well from row A to H. So, total volume in each well becomes 300 μL. Final sample concentration for wells in each row is such as following:

A: 100 μg/mL containing 0.2% DMSO

B: 50 μg/mL containing 0.2% DMSO

C: 25 μg/mL containing 0.2% DMSO

D: 12.5 µg/mL containing 0.2% DMSO

E: 6.25 µg/mL containing 0.2% DMSO

F: 3.13 µg/mL containing 0.2% DMSO

G: 1.56 µg/mL containing 0.2% DMSO

H: diluent Y

For ethacrynic acid, the preparation of dilution was done as previously described for plant sample. While for the doxorubicin hydrochloride, the stock was prepared at the 10 mg/mL in 100% DMSO. To prepare 1 mL of doxorubicin hydrochloride sub-stock at concentration of 20 µg/mL containing 0.2% DMSO, 2 µL of stock was added to 998 µL of diluent X. The serial dilution was done as previously described for plant sample preparation.

For cisplatin, the stock was prepared at 10 mg/mL in PBS. To prepare 1 mL of sub-stock at the concentration of 20 µg/mL, 2 µL of stock was added to 998 µL of diluent X. Serial dilution was started with the addition of 150 µL of diluent Z to wells in row B and addition of 150 µL of diluent Y to wells in row C to H (Appendix III, Figure 7.1). Then, 300 µL of sub-stock was added to wells in row A, at which the concentration of sample was 20 µg/mL. After that, 150 µL of solution in wells of row A was transferred to wells in row B, which create a second concentration at row B, which is 10 µg/mL. The dilution was continued until row G and 150 µL of solution in wells of row G was discarded. Hence, the volume in each well was 150 µL containing 0.2% DMSO. This is then followed by addition of 150 µL of diluent Y into every single well from row A to H. So, total volume in each well becomes 300 µL. Final sample concentration for wells in each row is such as following:

A: 10 μg/mL containing 0.2% DMSO

B: 5 μg/mL containing 0.2% DMSO

C: 2.5 μg/mL containing 0.2% DMSO

D: 1.25 μg/mL containing 0.2% DMSO

E: 0.625 μg/mL containing 0.2% DMSO

F: 0.313 μg/mL containing 0.2% DMSO

G: 0.156 μg/mL containing 0.2% DMSO

H: diluent Y

3.2.9.5 CELLS TREATMENT

After 24 h incubation, utilized media in each well of cell seeding plate was discarded with pipettor. Then, the wells in row A to G were replaced with 200 μL of sample-containing media prepared by serial dilution. The wells in row H was replaced with 200 μL of diluent Y. The final DMSO concentration in each well was 0.2%. Cells were then incubated for 72 h in humidified 5% CO_2 incubator at 37 ℃. The actual assay condition was illustrated in Appendix III, Figure 7.2 and 7.3.

Control groups in this assay include blank, cells in media containing 0.2% DMSO (negative control) and cells in media containing 0.156-10.0 μg/mL of doxorubicin hydrochloride or cisplatin (positive control).

3.2.9.6 CELL VIABILITY DETERMINATION

The cytotoxicity activities of samples were evaluated using MTT assay described by Mosmann (1983). After 72 h incubation, old media were discarded and 100 µL of 10% supplemented EMEM was dispensed into each well of cells. 20 µL of 5 mg/mL of MTT reagent was then added into each well and incubated for 4 h.

After 4 h of incubation time, the media were discarded and the formazan product of MTT reduction was dissolved in 100 µL of DMSO. The plate shook on a microplate mixer for 15 minutes. The optical density at 570 nm (OD_{570}) with reference wavelength of 650 nm, was determined using Asys HiTech UVM 340 microplate reader (Biochrom). The absorbance of formazan in negative control was taken as 100% viability. Cytotoxicity of each sample was expressed as IC_{50} value, which is the concentration of sample required to reduce the viability of cells by 50% compared to control (untreated cells).

3.2.10 CELL PROLIFERATION AND VIABILITY ASSAY

3.2.10.1 PREPARATION OF DILUENTS

The diluents used in this experiment were prepared as stated in Appendix II.

3.2.10.2 CELL CULTURE

The human colon adenocarcinoma cell line, HT-29, used in this experiment were cultured in 10% supplemented RPMI 1640 medium. Cell culture was maintained in humidified 5% CO_2 incubator at 37 ℃. The cells were grown to 90-100% confluence before proceed to seeding.

3.2.10.3 CELL PREPARATION AND SEEDING

Cytotoxicity effect of plant samples (ethanolic extract/fraction), doxorubicin hydrochloride, cisplatin and ethacrynic acid against HT-29 cells were tested. Monolayer HT-29 cells with 90-100% confluence were detached using accutase solution and transferred into a centrifuge tube containing 1mL of diluent A. The cells were spun at 1000 rpm for 5 minutes. Then, the supernatant was discarded. 1 mL of diluent A was added and the cells were gently resuspended.

10 µL of cell suspension was mixed with 90 µL of trypan blue solution in a microcentrifuge tube and then 10 µL of the mixture was transferred to a haemocytometer for cell counting. Percentage of cell viability was determined and viability should be at least 95% in order to proceed with the assay.

Cells were seeded in flat bottom 96-well microplate (Nunc, Denmark), at a concentration of 6 x 10^4 cells/mL, in 100 µL culture medium per well. Cells were allowed to attach for 24 h in humidified 5% CO_2 incubator at 37 ℃.

3.2.10.4 SAMPLE DILUTION PREPARATION

Sample stock and sub-stock were prepared before conducting serial dilution. Sample stock was prepared by dissolving 0.02 g of plant sample (ethanolic extract/fraction) in 0.2 mL of 100% DMSO, which final concentration is 100 mg/mL. Meanwhile, to prepare 1 ml of sub-stock at a concentration of 200 µg/mL containing 0.2% DMSO, 2 µL of stock was added to 998 µL of diluent A.

After preparation of sub-stock, serial dilution was conducted using flat bottom 96-well microplate and multi-channel pipettor. First of all, 150 µL of diluent B was added to wells in row B to H (Appendix III, Figure 7.1). Then, 300 µL of sub-stock was

added to wells in row A, at which the concentration of sample was 200 μg/mL. After that, 150 μL of solution in wells of row A was transferred to wells in row B, which create a second concentration at row B, which is 100 μg/mL. The dilution was continued until row G and 150 μL of solution in wells of row G was discarded. Hence, the volume in each well was 150 μL containing 0.2% DMSO. This is then followed by addition of 150 μL of diluent B into every single well from row A to H. So, total volume in each well becomes 300 μL. Final sample concentration for wells in each row is such as following:

A: 100 μg/mL containing 0.2% DMSO

B: 50 μg/mL containing 0.2% DMSO

C: 25 μg/mL containing 0.2% DMSO

D: 12.5 μg/mL containing 0.2% DMSO

E: 6.25 μg/mL containing 0.2% DMSO

F: 3.13 μg/mL containing 0.2% DMSO

G: 1.56 μg/mL containing 0.2% DMSO

H: diluent B

For ethacrynic acid, the preparation of dilution was done as previously described for plant sample. While for the doxorubicin hydrochloride, the stock was prepared at the 10 mg/mL in 100% DMSO. To prepare 1 mL of doxorubicin hydrochloride sub-stock at concentration of 20 μg/mL containing 0.2% DMSO, 2 μL of stock was added to 998 μL of diluent A. The serial dilution was done as previously described for plant sample preparation.

For cisplatin, the stock was prepared at 10 mg/mL in PBS. To prepare 1 mL of sub-stock at concentration of 20 μg/mL, 2 μL of stock was added to 998 μL of diluent A. Serial dilution was started with the addition of 150 μL of diluent C to wells in row B and addition of 150 μL of diluent B to wells in row C to H (Appendix III, Figure 7.1). Then, 300 μL of sub-stock was added to wells in row A, at which the concentration of sample was 20 μg/mL. After that, 150 μL of solution in wells of row A was transferred to wells in row B, which create a second concentration at row B, which is 10 μg/mL. The dilution was continued until row G and 150 μL of solution in wells of row G was discarded. Hence, the volume in each well was 150 μL containing 0.2% DMSO. This is then followed by addition of 150 μL of diluent B into every single well from row A to H. So, total volume in each well becomes 300 μL. Final sample concentration for wells in each row is such as following:

A: 10 μg/mL containing 0.2% DMSO

B: 5 μg/mL containing 0.2% DMSO

C: 2.5 μg/mL containing 0.2% DMSO

D: 1.25 μg/mL containing 0.2% DMSO

E: 0.625 μg/mL containing 0.2% DMSO

F: 0.313 μg/mL containing 0.2% DMSO

G: 0.156 μg/mL containing 0.2% DMSO

H: diluent B

3.2.10.5 CELLS TREATMENT

After 24 h incubation, utilized media in each well of cell seeding plate was discarded with pipettor. Then, the wells in row A to G were replaced with 200 µL of sample-containing media prepared by serial dilution. The wells in row H was replaced with 200 µL of diluent B. The final DMSO concentration in each well was 0.2%. Cells were then incubated for 72 h in humidified 5% CO_2 incubator at 37 ℃. The actual assay condition was illustrated in Appendix III, Figure 7.2 and 7.3.

Control groups in this assay include blank, cells in media containing 0.2% DMSO (negative control) and cells in media containing 0.156-10 µg/mL of doxorubicin hydrochloride or cisplatin (positive control).

3.2.10.6 CELL VIABILITY DETERMINATION

The cytotoxicity activities of samples were evaluated using MTT assay described by Mosmann (1983). After 72 h incubation, old media were discarded and 100 µL of 10% supplemented RPMI 1640 medium was added into each well of cells. 20 µL of 5 mg/mL of MTT reagent was then added into each well and incubated for 4 h.

After 4 h of incubation time, the media were discarded and the formazan product of MTT reduction was dissolved in 100 µL of DMSO. The plate shook on microplate mixer for 15 minutes. The optical density at 570 nm (OD_{570}) with reference wavelength of 650 nm, was determined using Asys HiTech UVM 340 microplate reader (Biochrom). The absorbance of formazan in negative control was taken as 100% viability. Cytotoxicity of each sample was expressed as IC_{50} value, which is the concentration of sample required to reduce the viability of cells by 50% compared to control (untreated cells).

3.2.11 DOXORUBICIN HYDROCHLORIDE AND PLANT SAMPLE COMBINATION CYTOTOXICITY ASSAY

In order to examine the synergic effect of doxorubicin hydrochloride and plant samples (ethanolic extract/fraction) on HT-29 cytotoxicity, doxorubicin hydrochloride at different concentrations in combination with fixed concentration of plant sample were tested against HT-29 cells.

Based on the results obtained from previous experiment (Section 3.2.10), plant sample concentration was fixed at 50 μg/mL and ethacrynic acid concentration (as a reference) was fixed at 10 μg/mL. The concentration fixed was the concentration at which viability of HT-29 cells is higher than 90%, when treated with samples.

3.2.11.1 PREPARATION OF DILUENTS

The diluents used in this experiment were prepared as stated in Appendix II.

3.2.11.2 CELL CULTURE

The human colon adenocarcinoma cell line, HT-29, used in this experiment were cultured in 10% supplemented RPMI 1640 medium. Cell culture was maintained in humidified 5% CO_2 incubator at 37℃. The cells were grown to 90-100% confluence before proceed to seeding.

3.2.11.3 CELL PREPARATION AND SEEDING

Cytotoxicity effect of doxorubicin hydrochloride in combination with ethacrynic acid and plant samples (ethanolic extract/fraction) against HT-29 cells were tested. Monolayer HT-29 cells with 90-100% confluence were detached using accutase solution and transferred into a centrifuge tube containing 1 mL of diluent A. The cells were spun at 1000 rpm for 5 minutes. Then, the supernatant was discarded. 1 mL of diluent A was added and the cells were gently resuspended.

10 µL of cell suspension was mixed with 90 µL of trypan blue solution in a microcentrifuge tube and then 10 µL of the mixture was transferred to a haemocytometer for cell counting. Percentage of cell viability was determined and viability should be at least 95% in order to proceed with the assay.

Cells were seeded in flat bottom 96-well microplate at a concentration of 6×10^4 cells/mL, in 100 µL culture medium per well. Cells were allowed to attach for 24 h in humidified 5% CO_2 incubator at 37 ℃.

3.2.11.4 SAMPLE DILUTION PREPARATION

Sample stock was prepared by dissolving 0.01 g of plant sample (ethanolic extract/fraction) in 0.2 mL of 100% DMSO, which final concentration is 50 mg/mL. Meanwhile, to prepare 1 mL of sub-stock at a concentration of 100 µg/mL containing 0.2% DMSO, 2 µL of stock was added to 998 µL of diluent A.

Ethacrynic acid stock was prepared at the 10 mg/mL in 100% DMSO. To prepare 1 mL of ethacrynic acid sub-stock at concentration of 20 µg/mL containing 0.2% DMSO, 2 µL of stock was added to 998 µL of diluent A.

For doxorubicin hydrochloride, the stock was prepared at the 10 mg/mL in 100% DMSO. To prepare 1 mL of doxorubicin hydrochloride sub-stock at concentration of 20 μg/mL containing 0.2% DMSO, 2 μL of stock was added to 998 μL of diluent A. After preparation of sub-stock, serial dilution was conducted using flat bottom 96-well microplate and multi-channel pipettor. First of all, 150 μL of diluent B was added to wells in row B to H (Appendix III, Figure 7.4). Then, 300 μL of sub-stock was added to wells in row A, at which the concentration of doxorubicin hydrochloride was 20 μg/mL. After that, 150 μL of solution in wells of row A was transferred to wells in row B, which create a second concentration at row B, which is 10 μg/mL. The dilution was continued until row G and 150 μL of solution in wells of row G was discarded. Hence, the volume in each well was 150 μL containing 0.2% DMSO. This is then followed by addition of 150 μL of plant sample sub-stock into every single well from row A to G. While for wells in row H, 150 μL of diluent B was added. So, total volume in each well becomes 300 μL. Final sample concentration for wells in each row is such as following:

A: 10 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

B: 5 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

C: 2.5 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

D: 1.25 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

E: 0.625 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

F: 0.313 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

G: 0.156 μg/mL doxorubicin hydrochloride + 50 μg/mL plant sample containing 0.2% DMSO

H: diluent B

With ethacrynic acid used as reference, 150 μL of ethacrynic acid sub-stock was added into every single well from row A to G (Appendix III, Figure 7.4) of serially diluted doxorubicin hydrochloride. The final concentration of ethacrynic acid in each well of dilution was 10 μg/mL, containing 0.2% DMSO.

3.2.11.5 CELLS TREATMENT

After 24 h incubation, utilized media in each well of cell seeding plate was discarded with pipettor. Then, the wells in row A to G were replaced with 200 μL of sample-containing media prepared by serial dilution. The wells in row H was replaced with 200 μL of diluent B. The final DMSO concentration in each well was 0.2%. Cells were then incubated for 72 h in humidified 5% CO_2 incubator at 37 ℃. The actual assay condition was illustrated in Appendix III, Figure 7.5.

Control groups in this assay include blank, cells in media containing 0.2% DMSO (negative control) and cells in media containing 0.156-10 μg/mL of doxorubicin hydrochloride (positive control).

3.2.11.6 CELL VIABILITY DETERMINATION

The cytotoxicity activities of the combination of doxorubicin hydrochloride and plant samples (ethanolic extract/fraction) were evaluated using MTT assay described by Mosmann (1983). After 72 h incubation, old media were discarded and 100 µL of 10% supplemented RPMI 1640 medium was added into each well of cells. 20 µL of 5 mg/mL of MTT reagent was then added into each well and incubated for 4 h.

After 4 h of incubation time, the media were discarded and the formazan product of MTT reduction was dissolved in 100 µL of DMSO. The plate was shook on a microplate mixer for 15 minutes. The optical density at 570 nm (OD_{570}) with reference wavelength of 650 nm, was determined using Asys HiTech UVM 340 microplate reader (Biochrom). The absorbance of formazan in negative control was taken as 100% viability. Cytotoxicity of each combination was expressed as IC_{50} value, which is the concentration of doxorubicin hydrochloride required to reduce the viability of cells by 50% compared to control (untreated cells).

3.2.12 CISPLATIN AND PLANT SAMPLE COMBINATION CYTOTOXICITY ASSAY

In order to examine the synergic effect of cisplatin and plant samples (ethanolic extract/fraction) on HT-29 cytotoxicity, cisplatin at different concentrations in combination with fixed concentration of plant sample were tested against HT-29 cells.

Based on the results obtained from previous experiment (Section 3.2.10), plant sample concentration was fixed at 50 µg/mL and ethacrynic acid concentration (as a reference) was fixed at 10 µg/mL. The concentration fixed was the concentration at which viability of HT-29 cells is higher than 90%, when treated with samples.

3.2.12.1 PREPARATION OF DILUENTS

The diluents used in this experiment were prepared as stated in Appendix II.

3.2.12.2 CELL CULTURE

The human colon adenocarcinoma cell line, HT-29, used in this experiment were cultured in 10% supplemented RPMI 1640 medium. Cell culture was maintained in humidified 5% CO_2 incubator at 37 ℃. The cells were grown to 90-100% confluence before proceed to seeding.

3.2.12.3 CELL PREPARATION AND SEEDING

Cytotoxicity effect of cisplatin in combination with ethacrynic acid and plant samples (ethanolic extract/fraction) against HT-29 cells were tested. Monolayer HT-29 cells with 90-100% confluence were detached using accutase solution and transferred into a centrifuge tube containing 1 mL of diluent A. The cells were spun at 1000 rpm for 5 minutes. Then, the supernatant was discarded. 1 mL of diluent A was added and the cells were gently resuspended.

10 µL of cell suspension was mixed with 90 µL of trypan blue solution in a microcentrifuge tube and then 10 µL of the mixture was transferred to a haemocytometer for cell counting. Percentage of cell viability was determined and viability should be at least 95% in order to proceed with the assay.

Cells were seeded in flat bottom 96-well microplate at a concentration of 6×10^4 cells/mL, in 100 µL culture medium per well. Cells were allowed to attach for 24 h in humidified 5% CO_2 incubator at 37 ℃.

3.2.12.4 SAMPLE DILUTION PREPARATION

Sample stock was prepared by dissolving 0.01 g of plant sample (ethanolic extract/fraction) in 0.2 mL of 100% DMSO, which final concentration is 50 mg/mL. Meanwhile, to prepare 1 mL of sub-stock at a concentration of 100 μg/mL containing 0.2% DMSO, 2 μL of stock was added to 998 μL of diluent A.

Ethacrynic acid stock was prepared at the 10 mg/mL in 100% DMSO. To prepare 1 mL of ethacrynic acid sub-stock at concentration of 20 μg/mL containing 0.2% DMSO, 2 μL of stock was added to 998 μL of diluent A.

For cisplatin, the stock was prepared at 10 mg/mL in PBS. To prepare 1 mL of sub-stock at concentration of 20 μg/mL, 2 μL of stock was added to 998 μL of diluent A. After preparation of sub-stock, serial dilution was conducted using flat bottom 96-well microplate and multi-channel pipettor. Serial dilution was started with the addition of 150 μL of diluent C to wells in row B and addition of 150 μL of diluent B to wells in row C to H (Appendix III, Figure 7.4). Then, 300 μL of sub-stock was added to wells in row A, at which the concentration of sample was 20 μg/mL. After that, 150 μL of solution in wells of row A was transferred to wells in row B, which create a second concentration at row B, which is 10 μg/mL. The dilution was continued until row G and 150 μL of solution in wells of row G was discarded. Hence, the volume in each well was 150 μL containing 0.2% DMSO. This is then followed by addition of 150 μL of plant sample sub-stock into every single well from row A to G. While for wells in row H, 150 μL of diluent B was added. So, total volume in each well becomes 300 μL. Final sample concentration for wells in each row is such as following:

A: 10 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

B: 5 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

C: 2.5 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

D: 1.25 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

E: 0.625 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

F: 0.313 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

G: 0.156 μg/mL cisplatin + 50 μg/mL plant sample containing 0.2% DMSO

H: diluent B

With ethacrynic acid used as reference, 150 μL of ethacrynic acid sub-stock was added into every single well from row A to G (Appendix III, Figure 7.4) of serially diluted cisplatin. The final concentration of ethacrynic acid in each well of dilution was 10 μg/mL, containing 0.2% DMSO.

3.2.12.5 CELLS TREATMENT

After 24 h incubation, utilized media in each well of cell seeding plate was discarded with pipettor. Then, the wells in row A to G were replaced with 200 μL of sample-containing media prepared by serial dilution. The wells in row H was replaced with 200 μL of diluent B. The final DMSO concentration in each well was 0.2%. Cells were then incubated for 72 h in humidified 5% CO_2 incubator at 37 ℃. The actual assay condition was illustrated in Appendix III, Figure 7.6.

Control groups in this assay include blank, cells in media containing 0.2% DMSO (negative control) and cells in media containing 0.156-10 μg/mL of cisplatin (positive control).

3.2.12.6 CELL VIABILITY DETERMINATION

The cytotoxicity activities of the combination of cisplatin and plant samples (ethanolic extract/fraction) were evaluated using MTT assay described by Mosmann (1983). After 72 h incubation, old media were discarded and 100 μL of 10% supplemented RPMI 1640 medium was added into each well of cells. 20 μL of 5 mg/mL of MTT reagent was then added into each well and incubated for 4 h.

After 4 h of incubation time, the media were discarded and the formazan product of MTT reduction was dissolved in 100 μL of DMSO. The plate shook on a microplate mixer for 15 minutes. The optical density at 570 nm (OD_{570}) with reference wavelength of 650 nm, was determined using Asys HiTech UVM 340 microplate reader (Biochrom). The absorbance of formazan in negative control was taken as 100% viability. Cytotoxicity of each combination was expressed as IC_{50} value, which is the concentration of cisplatin required to reduce the viability of cells by 50% compared to control (untreated cells).

3.2.13 STATISTICAL ANALYSIS

All experiments were performed in three triplicates (N=3). The data were presented as mean ± standard deviation (S.D.) and were statistically analysed with SigmaPlot 12.0 graph and analysis software.

IC_{50} values of GST-P inhibitory assays and cytotoxicity assays were obtained from dose-response graph generated using Microsoft Excel software.

4.0 RESULTS

4.1 HT-29 CELL LINE

In my study, human HT-29 colon adenocarcinoma cell line is selected for the extraction of enzyme Glutathione S-Transferase π (GST-P), which will then react with selected plant sample (ethanolic extract/fraction) in GST-P inhibitory assays. HT-29 is selected due to its high expression of GST-P (Tashiro *et al*., 2001). The images of the monolayer HT-29 cells cultured in conventional RPMI 1640 medium are shown in Figure 4.1-4.3 at the objective power of 5X, 10X and 20X.

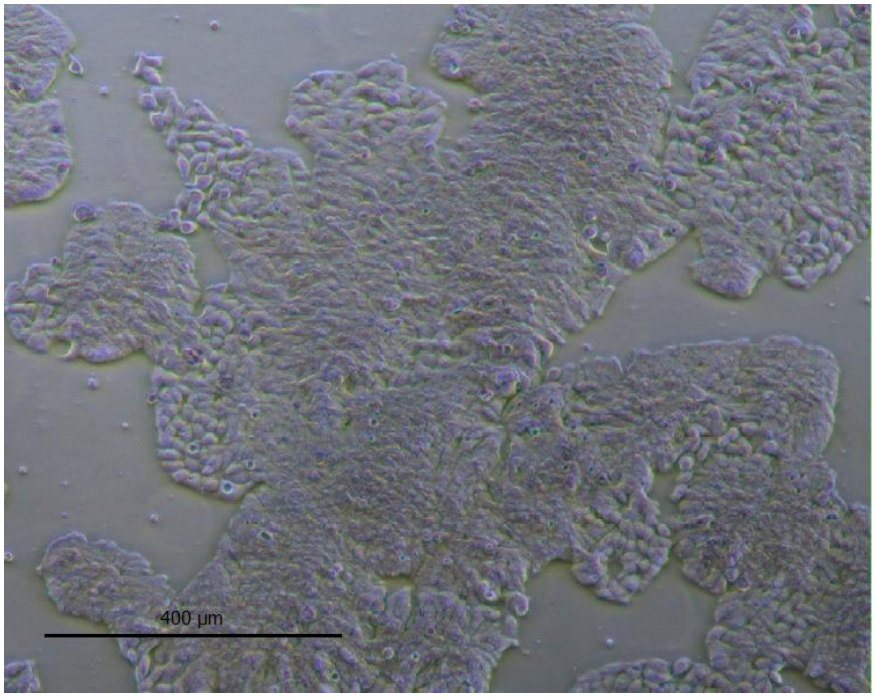

Figure 4.1 : Monolayer HT-29 cells cultured in 10% supplemented RPMI 1640 medium. 5X objective.

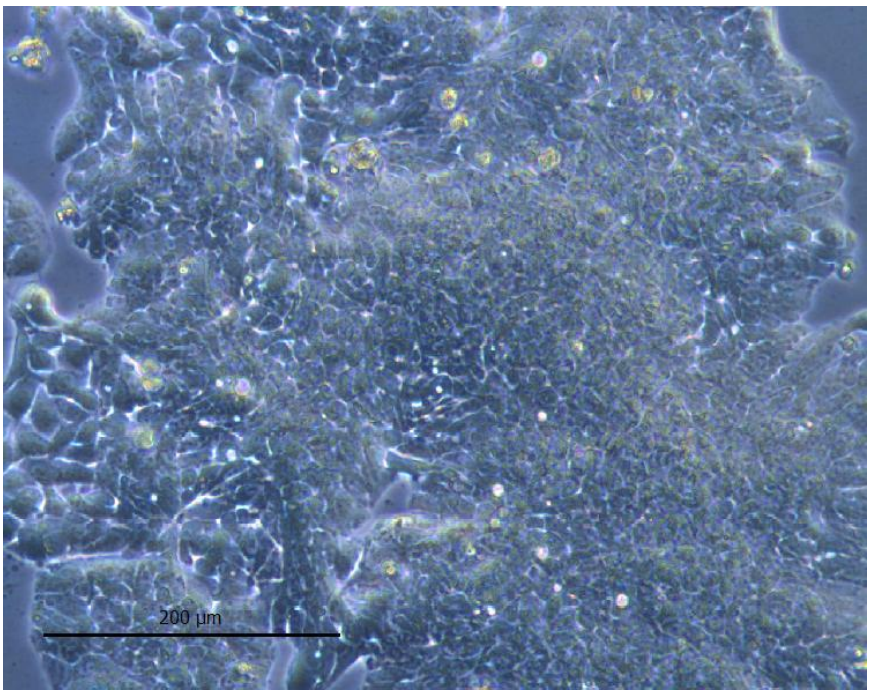

Figure 4.2 : Subconfluent culture of monolayer HT-29 cells. 10X objective.

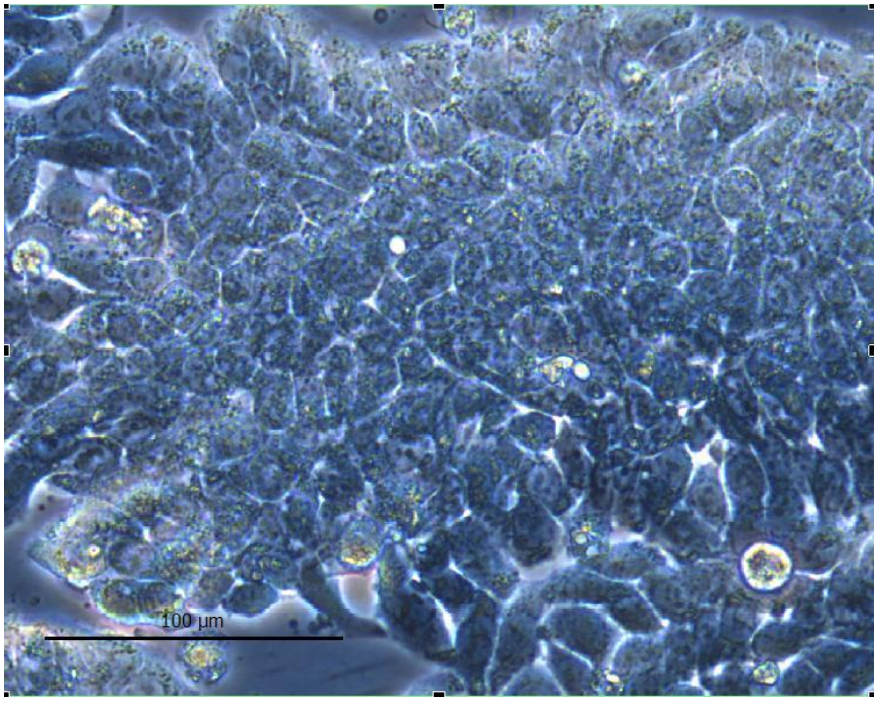

Figure 4.3 : Morphology of monolayer HT-29 cells. 20X objective.

HT-29 cell is a type of adherent cell which will adhere to surface of the culture flask once seeded. It is grown as monolayer and it acquired an epithelial-like morphology. Under standard culture condition, HT-29 cells grew into compact cell clusters with distinct intercellular cysts (Figure 4.1-4.3). When the cell growth reached 90-100% confluent, the cells were lysed and the lysate was spun at 15,000 xg to remove the cell debris. Clear lysate was subjected to affinity chromatography for the extraction of GST protein.

4.2 AFFINITY CHROMATOGRAPHY

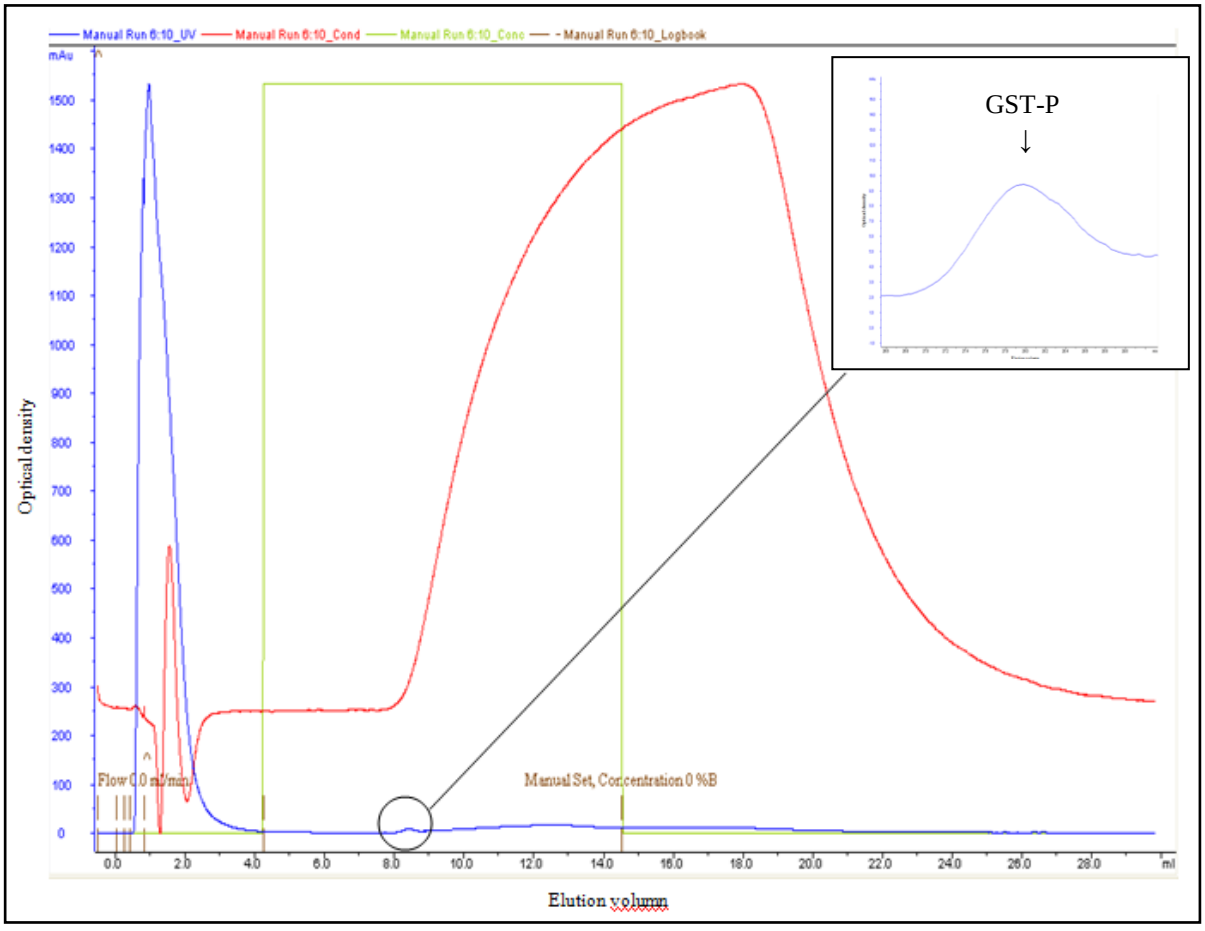

Figure 4.4 : Protein elution profile obtained for affinity chromatography on GSTrap™ HP column. The insert shows a magnification of the elution peak of purified GST-P protein.

Figure 4.4 shows the elution profile of HT-29 cell lysate. The optical density (OD) shown by the blue line is the UV absorbance of proteins at the wavelength of 280 nm. The red line represented the conductivity of the chromatography. The first peak indicates the elution of unbound proteins which does not bind to the GST affinity column. The unbound protein eluted out with the 25 mM sodium phosphate buffer, pH 7.4, after the sample injected into the system. There is not GST enzyme detected in the unbound fraction as there was no GST activity measured with CDNB (result not shown). That means, all of the GST has been bound to column. When the unbound protein has been completely eluted, the buffer was then changed to the 10 mM reduced glutathione in 25 mM sodium phosphate buffer, pH 7.4, which has high affinity for GST. The second peak which has been highlighted in the insert is the peak for GST protein which has been eluted out together with the reduced GSH. The GST eluate has shown a high GST activity when reacted with CDNB and reduced GSH. The following peak is the peak for reduced GSH. From the insert, we can see that the amount of GST obtained is very little as there was only 1 mL of lysate has been injected.

4.3 GLUTATHIONE S-TRANSFERASE π IDENTIFICATION

4.3.1 GST ACTIVITY

The activity of the GST isolated from HT-29 cell line was determined with spectrophotometric enzyme assay described by Habig *et al.* (1974). CDNB and reduced GSH were used as substrates. With GST protein of 10 mg (weighed lyophilized GST) in 1 mL buffer A, the total activity obtained was 0.3228 μmol/min. Thus, the specific activity of the GST is 0.03228 μmol/min/mg protein.

4.3.2 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE is one of the molecular biology techniques that frequently used to separate protein accordingly by size. It is probably the most powerful technique used for resolving protein mixtures. The ionic detergent, sodium dodecylsulfate (SDS), denatures the protein and dissociates multimeric proteins into their respective subunits. Besides, all of the polypeptide chains are in the conformation with similar charge over mass ratio and thus chain length which reflects the mass, is the sole determinant of the migration rate.

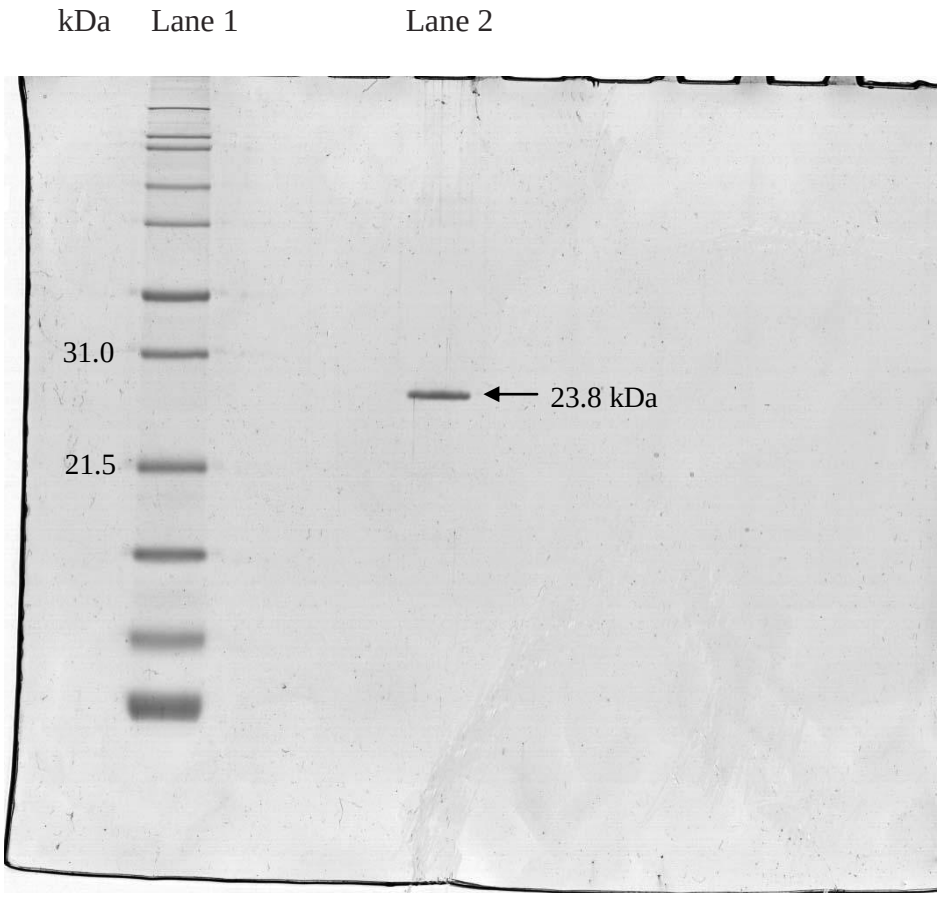

Figure 4.5 : SDS-PAGE banding pattern of purified GST-P from HT-29 cells.
Lane 1: Mark 12 unstained protein standard markers.
Lane 2: Concentrated eluent of GST obtained from affinity chromatography.

By referring to Figure 4.5, there was only single protein band observed in lane 2 which loaded with concentrated GST eluent obtained from affinity chromatography. The single band was positioned in between the bands of carbonic anhydrase and trypsin inhibitor, which molecular weight are 31.0 and 21.5 kDa respectively. That mean, the protein present is the monomers of an isoform of GST which has a range of monomer molecular weight in between 25 and 30 kDa.

To further determine the actual molecular weight of the GST isoform, a standard curve of log molecular weight against relative mobility is constructed (Appendix V, Figure 7.85). The standard curve is constructed based on the molecular weight of the proteins used as standard marker in Mark™ 12 unstained marker and the relative mobility, R_f, of each proteins which can be calculated by the division of the distance migrated of the protein band over the distance migrated by the solvent front on SDS-PAGE gel. Then, the molecular weight of the GST was determined from the curve by comparing the R_f of the GST band with the curve. The calculated R_f value for the GST band was 0.51. Thus, the molecular weight of the GST obtained was 23.8 kDa and this is near to the molecular weight of human GST-P as reported by Aliya *et al*. (2003). To further confirm with this, the protein band was then subjected to peptide mass fingerprint analysis.

4.3.3 PEPTIDE MASS FINGERPRINT (PMF) ANALYSIS

The need for highly accurate and sensitive methods to analyse biomolecules are increasingly important due to the increase in interdisciplinary research. Mass spectrometry (MS) has becomes an important tool for the characterization and analysis of variant complex biomolecules, especially the matrix assisted laser desorption/ionization (MALDI) mass spectrometry which able to analyze biomolecules

up to over 300kDa (Karas *et al*., 1987). Meanwhile, the matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry has improved mass resolution obtained by utilization of either single-stage or a dual-stage reflectron (RETOF-MS).

The result obtained from PMF analysis (Appendix IV) indicates that the purified GST is belongs to the human GST-P (EC 2.5.1.18). The nominal mass (M_r) is 23341 Da and the calculated pI valus is 5.43. The result convinced that HT-29 cell line expressed high level of GST-P isoform and this is compatible with data obtained from Schultz *et al*., (1997) that only mRNA of GST-P quantified from HT-29 cell line.

4.3.4 ISOELECTRIC FOCUSING (IEF) ELECTROPHORESIS

In order to determine the number of pi isoform of GST exist in the purified GST sample, IEF was carried out. IEF electrophoresis separated proteins according to their isoelectric point (pI) based on the fact that overall charge on the protein molecule is a function of its surrounding pH. Figure 4.6 shows the image of protein bands on a pre-cast IEF mini gel. The image was captured with ImageScanner III (GE Healthcare).

By referring to Figure 4.6, there was only single protein band observed in lane 2 and 3 which loaded with concentrated GST eluent obtained from affinity chromatography. The single band was positioned in between the bands of β-lactoglobulin and carbonic anhydrase, which pI values are 5.3 and 6.0 respectively. That mean, the protein present is the monomers of single isoform of GST-P and the expected pI value is 5.4. So, there is only one isoform of GST-P purified from HT-29 cell line.

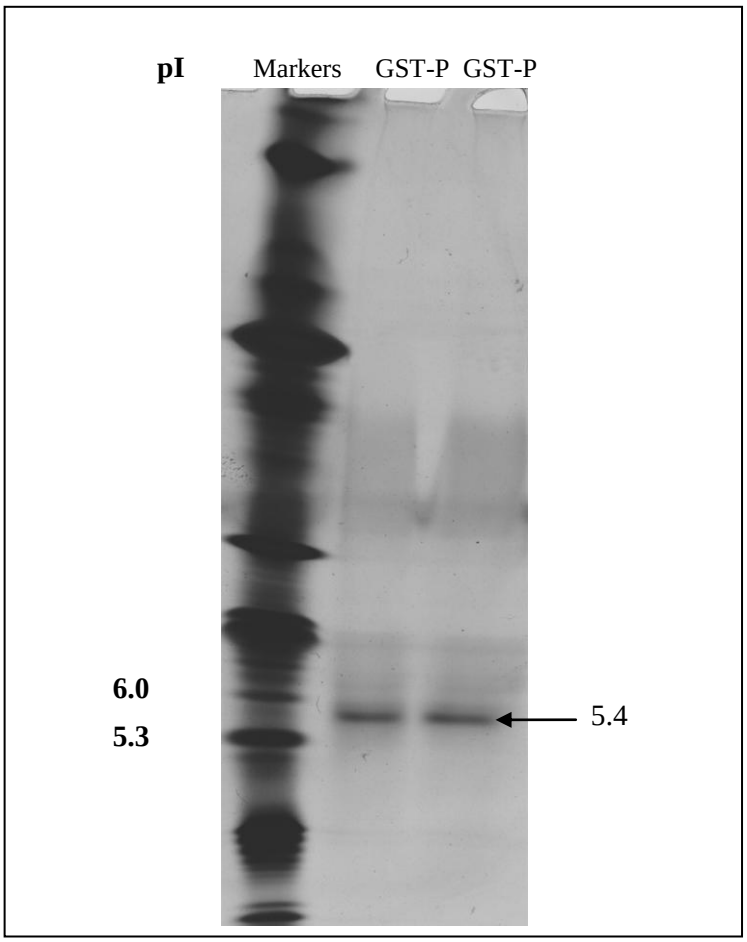

Figure 4.6 : Analytical isoelectric focusing (IEF) of purified GST-P. IEF gel showing the pI value of the replicate of purified GST-P loaded at lane 2 and 3. Lane 1 is loaded with SERVA IEF markers 3-10.

4.4 PRELIMINARY SCREENING ON GST-P INHIBITION

This preliminary study was done to establish the inhibitory effects of local plants on GST-P. 43 plant ethanolic extracts have been screened for possible inhibitor of GST-P. However, some of them appeared to be the enhancer of GST-P instead of inhibitor based on the results obtained in Table 4.1. The effects of ethanolic extracts on GST-P were determined by relative changes in specific activity of GST-P with the addition of the ethanolic extracts.

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Table 4.1 : Relative changes of GST-P specific activity by ethanolic extracts of local plants

Sample	Relative changes in specific activity of GST-P, (%)
Anacardium occidentale (Gajus)-branch	↓ 64
Anacardium occidentale (Gajus)-fruit & flower	↓ 8
Andrographis paniculata (Hempedu bumi)-branch	↓ 36
Andrographis paniculata (Hempedu bumi)-leaf	↓ 61
Artocarpus heterophyllus (Nangka)-leaf	↑ 5
Averrhoa bilimbi (Belimbing buluh)-branch	↑ 20
Averrhoa bilimbi (Belimbing buluh)-leaf	↓ 17
Cinnamomum zeylanicum (kayu manis)-branch	↓ 78
Commelina nudiflora (Rumput kupu-kupu)-whole plant	↑ 5
Euodia redlevi (Tenggek burung)-flower & fruit	↓ 82
Eupatorium odoratum (Pokok jepun)-branch	↑ 51
Fagraea fragrans (Tembusu)- leaf & branch	↑ 18
Fagraea fragrans (Tembusu)-fruit & flower	↓ 43
Ficus auriculata (Ara)-leaf	↓ 50
Garcinia atroviridis (Asam gelugor)-branch	↓ 100
Garcinia mangostana (Manggis)-branch	↓ 72
Garcinia mangostana (Manggis)-leaf	↓ 75
Hibiscus tiliaceus (Bebaru)-leaf	↓ 81
Ipomoea aquatica (Kangkung)-leaf	↓ 17
Justicia gendarussa (Gandarusa, hitam)-branch	↑ 9
Lagerstroemia speciosa (Bungur)-fruit	↓ 50
Lagerstroemia speciosa (Bungur)-leaf	↓ 44
Lawsonia inermis (Inai)-branch	↓ 52
Leptospermum flavescens (Gelam bukit)-leaf	↓ 100
Leptospermum flavescens (Gelam bukit)-branch	↓ 46
Macaranga conifera (Mahang)-branch	↓ 31
Melaleuca cajuputi (Gelam)-fruit & flower	↓ 4
Moringa oleifera (Kelor)-branch	↑ 66
Orthosiphon stamineus (Misai kucing)-leaf	↓ 15
Oxalis barrelieri (Belimbing tanah)-root	↓ 75
Peltophorum pterocarpum (Batai laut)-fruit	↓ 24
Peltophorum pterocarpum (Batai laut)-leaf	↓ 72
Pereskia bleo (Jarum 7 bilah)-fruit	↓ 10
Piper nigrum (Lada hitam)- leaf	↑ 27
Piper nigrum (Lada hitam)-branch	↓ 21
Piper nigrum (Lada hitam)-fruit	↑ 97
Scoparia dulcis (Rempah padang)-root	↑ 17
Strobilanthes crispa (Bayam karang)-branch	↑ 14
Tetracera indica (Mempelas)-fruit	↓ 71
Vitex sp.-branch	↑ 87
Vitex sp.-leaf	↑ 12
Vitex trifolia 'purpurea' (Legundi)-branch	↓ 6
Vitex trifolia 'purpurea' (Legundi)-leaf	↓ 24

* ↑ = increase in specific activity of GST-P ↓= decrease in specific activity of GST-P

From Table 4.1, ethanolic extracts of *Anacardium occidentale* (branch and fruit&flower), *Andrographis paniculata* (branch and leaf), *Averrhoa bilimbi* (leaf), *Cinnamomum zeylanicum* (branch), *Euodia redlevi* (fruit&flower), *Fagraea fragrans* (fruit&flower), *Ficus auriculata* (leaf), *Garcinia atroviridis* (branch), *Garcinia mangostana* (branch and leaf), *Hibiscus tiliaceus* (leaf), *Ipomoea aquatica* (leaf), *Lagerstroemia speciosa* (leaf and fruit), *Lawsonia inermis* (branch), *Leptospermum flavescens* (branch and leaf), *Macaranga conifera* (branch), *Melaleuca cajuputi* (fruit&flower), *Orthosiphon stamineus* (leaf), *Oxalis barrelieri* (root), *Peltophorum pterocarpum* (leaf and fruit), *Pereskia bleo* (fruit), *Piper nigrum* (branch), *Tetracera indica* (fruit), *Vitex trifolia 'purpurea'* (branch and leaf) are those samples shown inhibition activity on GST-P by causing a decrease in specific activity of GST-P. However, *Anacardium occidentale* (branch) (64%), *Andrographis paniculata* (leaf) (61%), *Cinnamomum zeylanicum* (branch) (78%), *Euodia redlevi* (fruit&flower) (82%), *Garcinia atroviridis* (branch) (100%), *Garcinia mangostana* (branch and leaf) (72% and 75%), *Hibiscus tiliaceus* (leaf) (81%), *Lawsonia inermis* (branch) (52%), *Leptospermum flavescens* (leaf) (100%), *Oxalis barrelieri* (root) (75%), *Peltophorum pterocarpum* (leaf) (72%) and *Tetracera indica* (fruit) (71%) ethanolic extracts are more potent inhibitors as they have a relative reduction of more than 50%. These ethanolic extracts were then selected for the subsequent experiments. And, the most potent inhibitors which have 100% inhibition on GST-P are found to be the ethanolic extracts of *Garcinia atroviridis* (branch) and *Leptospermum flavescens* (leaf). Both ethanolic extracts were then further fractionated to identify the possible inhibitory peptides present in the samples.

In contrast to the inhibition effect, ethanolic extracts of *Artocarpus heterophyllus* (leaf) (5%), *Averrhoa bilimbi* (branch) (20%), *Commelina nudiflora* (whole plant) (5%), *Eupatorium odoratum* (branch) (51%), *Fagraea fragrans*

(branch&leaf) (18%), *Justicia gendarussa* (branch) (9%), *Moringa oleifera* (branch) (66%), *Piper nigrum* (leaf and fruit) (27% and 97%), *Scoparia dulcis* (root) (17%), *Strobilanthes crispa* (branch) (14%) and *Vitex* sp. (branch and leaf) (87% and 12%) increased GST-P activity. Some of them even have activity higher than 50%. However, they are not in our research interest which is looking for GST-P inhibitor. Anyway, these plants can be use as functional food to boost up body GST activity.

A comparative GST inhibition study carried out by using polyamide fractionated fractions of *L. flavescens* (leaf) and *G. atroviridis* (branch) ethanolic extracts with purified GST-P (Table 4.2). The results indicated that only 50% MeOH 2% acetic acid fractions of *L. flavescens* (leaf) and *G. atroviridis* (branch) ethanolic extracts showed inhibition on GST-P. The relative reduction percentage of 50% MeOH 2% acetic acid fractions of *L. flavescens* (leaf) and *G. atroviridis* (branch) ethanolic extract are 95% and 11% respectively. However, only 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract selected to continue with subsequent experiment since there is only little inhibition activity showed by 50% MeOH 2% acetic acid fraction of *G. atroviridis* (branch) ethanolic extract. The 2% acetic acid fractions for both *L. flavescens* (leaf) and *G. atroviridis* (branch) ethanolic extracts had no inhibition toward GST-P activity.

Table 4.2 : Relative changes of GST-P specific activity with fractions of *Leptospermum flavescens* (leaf) and *Garcinia atroviridis* (branch) ethanolic extracts

Fraction	**Relative reduction in specific activity of GST-P, (%)**
L. flavescens (leaf) ethanolic extract	
2% Acetic acid	0
50% MeOH 2% Acetic acid	95
G. atroviridis (branch) ethanolic extract	
2% Acetic acid	0
50% MeOH 2% Acetic acid	11

* MeOH = methanol

4.5 IC_{50} VALUES OF SAMPLES ON GST-P INHIBITION

There were 13 ethanolic extracts and 1 fraction of selected plant samples tested in this experiment. The concentration of GST-P is fixed at 0.2 mg/mL. GST-P inhibition effect at various concentrations of plant samples (ethanolic extract/fraction) was determined and the dose-response graph, percentage of inhibition on GST activity (%) versus concentration, of each sample was generated using the software Microsoft Excel (Appendix IV, Figure 7.7-7.20). ANOVA was done on each sample using the software of SigmaPlot 12.0.

The half maximal inhibitory concentration (IC_{50}) values are the concentration of samples where 50% of the GST-P enzyme activity is inhibited compared to controls run without addition of samples. The IC_{50} value of each sample (ethanolic extract/fraction) was determined from the dose-response graph. IC_{50} resulting from the inhibition of GST-P by ethanolic extracts and fraction were showed in Figure 4.7 and Table 4.3.

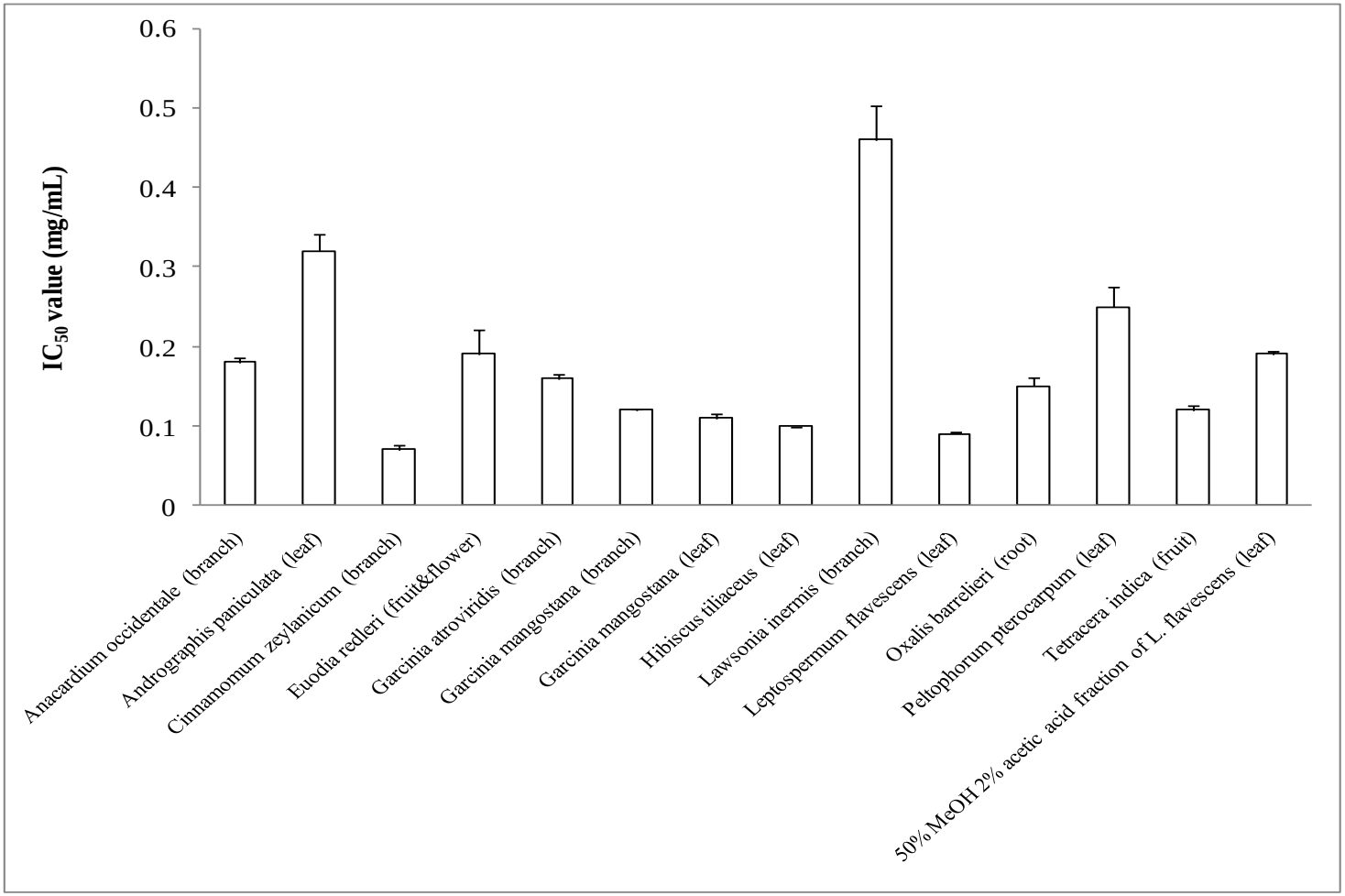

Figure 4.7 : IC_{50} values of selected samples in GST-P inhibitory assays. Each bar represents the mean ± S.D. calculated from three independent determinations. Comparison of IC_{50} values between samples using ANOVA indicates statistically significant difference with P = <0.001.

Table 4.3 : IC_{50} values of samples on GST-P

Plant sample	**IC_{50} (mean ± S.D., mg/mL)**	**Significant difference, P**
Ethanolic extract		
Anacardium occidentale (branch)	0.18 ± 0.006	<0.001
Andrographis paniculata (leaf)	0.32 ± 0.021	<0.001
Cinnamomum zeylanicum (branch)	0.07 ± 0.006	<0.001
Euodia redlevi (fruit&flower)	0.19 ± 0.031	<0.001
Garcinia atroviridis (branch)	0.16 ± 0.005	<0.001
Garcinia mangostana (branch)	0.12 ± 0.002	<0.001
Garcinia mangostana (leaf)	0.11 ± 0.006	<0.001
Hibiscus tiliaceus (leaf)	0.10 ± 0.000	<0.001
Lawsonia inermis (branch)	0.46 ± 0.042	<0.001
Leptospermum flavescens (leaf)	0.09 ± 0.002	<0.001
Oxalis barrelieri (root)	0.15 ± 0.012	<0.001
Peltophorum pterocarpum (leaf)	0.25 ± 0.025	<0.001
Tetracera indica (fruit)	0.12 ± 0.006	<0.001
Fraction		
50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract	0.19 ± 0.004	<0.001

* MeOH= Methanol. IC_{50} values indicated are the means (± standard deviation, S.D.) of three determinations.

In general, IC_{50} is a quantitative measurement of the effectiveness of a compound in inhibiting biological or biochemical function *in vitro* (FDA, 2000). The lower the IC_{50} value of a sample, the more effective the sample in inhibiting GST-P. From Table 4.3, Ethanolic extract of *Cinnamomum zeylanicum* (branch) has the lowest IC_{50} value, which is 0.07 mg/mL. That means Ethanolic extract of *Cinnamomum zeylanicum* (branch) is the most active sample in inhibiting GST-P activity. This is then followed by ethanolic extracts of *Leptospermum flavescens* (leaf), *Hibiscus tiliaceus* (leaf), *Garcinia mangostana* (leaf), *Garcinia mangostana* (branch) *Tetracera indica* (fruit), *Oxalis barrelieri* (root), *Garcinia atroviridis* (branch), *Anacardium occidentale* (branch), *Euodia redlevi* (fruit&flower) and 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract, which has an IC_{50} value of less than 0.2 mg/mL. *Peltophorum pterocarpum* (leaf), *Andrographis paniculata* (leaf) and *Lawsonia inermis* (branch) ethanolic extracts has an IC_{50} value slightly higher than 0.2 mg/mL, which is 0.25, 0.32 and 0.46 mg/mL respectively.

4.6 KINETIC STUDIES

In brief, enzyme kinetics in a biological system is always related with K_m and V_{max} values. K_m is the concentration of substrate which required to produce an enzyme reaction at 1/2 V_{max}. While for V_{max}, it is the maximum rate that the enzyme reaction can proceed at. Theoretically, when there is a competitive inhibition, K_m appears to increase but V_{max} remain the same; non-competitive inhibition, V_{max} decrease and K_m remain the same; uncompetitive inhibition, both K_m and V_{max} appear to decrease and for mixed inhibition, K_m increase but V_{max} decrease (Segel, 1993).

The nature of the inhibition of GST-P by selected plant samples (ethanolic extract/fraction) was studied. The activities of GST-P were measured with variable

concentrations of GSH (0.2-1.0 mM) with the presence of a fixed concentration of plant samples (ethanolic extract/fraction). The CDNB was maintained at 1 mM. V_{max} and K_m values of GST-P with and without addition of plant samples (ethanolic extract/fraction) were determined from Michaelis-Menten equation and Lineweaver-Burk plot generated using SigmaPlot 12.0 software. V_{max} and K_m values were shown in Table 4.4.

By referring to Table 4.4, almost all of plant samples (ethanolic extract/fraction) exhibit mixed mode inhibition on GST-P, except for ethanolic extracts of *Andrographis paniculata* (leaf), *Garcinia mangostana* (leaf) and *Lawsonia inermis* (branch) which shown uncompetitive inhibition, Most of plant samples (ethanolic extract/fraction) tested in this experiment displayed mixed mode inhibition because the samples are in the form of mixture and thus there were probably more than one type of GST-P inhibitor present in one single sample.

Table 4.4 : V_{max} and K_m values of GST-P in the presence of plant samples (ethanolic extract/fraction)

Sample (Concentration used, mg/mL)	**V_{max} (µmol/min)**	**K_m (mM)**	**Type of inhibition**
GST-P (0.2)	0.22	0.58	-
Ethanolic extract			
Anacardium occidentale (branch) (0.2)	0.05	7.47	Mixed
Andrographis paniculata (leaf) (0.3)	0.06	0.47	Uncompetitive
Cinnamomum zeylanicum (branch) (0.1)	0.05	0.99	Mixed
Euodia redlevi (fruit&flower) (0.2)	0.15	3.01	Mixed
Garcinia atroviridis (branch) (0.2)	0.16	4.51	Mixed
Garcinia mangostana (branch) (0.1)	0.05	0.89	Mixed
Garcinia mangostana (leaf) (0.1)	0.03	0.38	Uncompetitive
Hibiscus tiliaceus (leaf) (0.1)	0.04	0.91	Mixed
Lawsonia inermis (branch) (0.5)	0.04	0.40	Uncompetitive
Leptospermum flavescens (leaf) (0.1)	0.02	0.62	Mixed
Oxalis barrelieri (root) (0.2)	0.04	0.61	Mixed
Peltophorum pterocarpum (leaf) (0.3)	0.05	13.82	Mixed
Tetracera indica (fruit) (0.1)	0.06	1.18	Mixed
Fraction			
50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract (0.2)	0.08	6.29	Mixed

* MeOH= Methanol. All GST-P activity data were obtained from triplicate determinations.

4.7 TOXICITY EFFECTS OF SAMPLES ON MRC-5 CELLS

In order to examine the toxicity effects of selected plant samples (ethanolic extract/fraction) and drugs on normal cells, *in vitro* cytotoxicity effects of the samples against MRC-5 cell line were evaluated using MTT assay. The effect at different concentrations of samples was studied after 72 h treatment. The dose-response graph for each sample (Appendix IV, Figure 7.21-7.37) was generated using Microsoft Excel software. Statistical analysis of data obtained was done by using SigmaPlot 12.0 analytical software.

IC_{50} values of the samples, which obtained from the dose-response curve, were shown in Table 4.5. IC_{50} value is the concentration of sample that reduced the cell viability by 50% compared to control, which were treated with 0.2% DMSO. The percentage of cell viability at the highest concentration tested (100 μg/mL) for each plant sample (ethanolic extract/fraction) was also stated in Table 4.5.

From Table 4.5, all of the plant samples (ethanolic extract/fraction) show no toxicity to MRC-5 cells up to 50 μg/mL. *Anacardium occidentale* (branch), *Euodia redlevi* (fruit&flower), *Garcinia atroviridis* (branch), *Garcinia mangostana* (leaf), *Hibiscus tiliaceus* (leaf) and *Leptospermum flavescens* (leaf) ethanolic extracts were not cytotoxic to MRC-5 cells as they have a 100% cell viability when treated at the highest concentration of 100 μg/mL. Meanwhile, *Andrographis paniculata* (leaf), *Cinnamomum zeylanicum* (branch), *Garcinia mangostana* (branch), *Oxalis barrelieri* (root) and *Peltophorum pterocarpum* (leaf) ethanolic extracts and 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract shown less toxicity on MrC-5 cells, with cell viability ≥ 80% when treated at 100 μg/mL. Ethanolic extracts of *Lawsonia inermis* (branch) and *Tetracera indica* (fruit) were slightly toxic to MRC-5 cells with cell viability of 75% and 78% respectively, when treated at 100 μg/mL. Doxorubicin

hydrochloride is more toxic to MRC-5 cells as compared to cisplatin and ethacrynic acid as it has the lowest IC_{50} values, which is 0.82 ± 0.011 μg/mL.

Table 4.5 : Cytotoxicity effects of samples on MRC-5 cells

Sample	**IC_{50} (mean ± S.D., μg/mL)**	**Percentage of cell viability at 100 μg/mL (%)**
Doxorubicin hydrochloride	0.82 ± 0.011	ND
Cisplatin	4.07 ± 0.04	ND
Ethacrynic acid	22.71 ± 0.24	1
Ethanolic extract		
Anacardium occidentale (branch)	>100	100
Andrographis paniculata (leaf)	>100	94
Cinnamomum zeylanicum (branch)	>100	97
Euodia redlevi (fruit&flower)	>100	100
Garcinia atroviridis (branch)	>100	100
Garcinia mangostana (branch)	>100	80
Garcinia mangostana (leaf)	>100	100
Hibiscus tiliaceus (leaf)	>100	100
Lawsonia inermis (branch)	>100	75
Leptospermum flavescens (leaf)	>100	100
Oxalis barrelieri (root)	>100	88
Peltophorum pterocarpum (leaf)	>100	99
Tetracera indica (fruit)	>100	78
Fraction		
50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract	>100	97

* MeOH= Methanol, ND= not determined. IC_{50} values indicated are the means (± standard deviation, S.D.) of three independent experiments.

4.8 EFFECTS OF SAMPLES ON HT-29 CELL PROLIFERATION

In order to examine the effects of selected plant samples (ethanolic extract/fraction) and drugs on HT-29 cell viability and proliferation, *in vitro* cytotoxicity effects of the samples against HT-29 cell line were evaluated using MTT assay. The effect at different concentrations of samples was studied after 72 h treatment. The dose-response graph for each sample (Appendix IV, Figure 7.38-7.54) was generated using Microsoft Excel software. Statistical analysis of data obtained was done by using SigmaPlot 12.0 analytical software.

IC_{50} values of the samples, which obtained from the dose-response curve, were shown in Table 4.6. IC_{50} value is the concentration of sample that reduced the cell viability by 50% compared to control, which were treated with 0.2% DMSO. The percentage of cell viability at the highest concentration tested (100 μg/mL) for each plant sample (ethanolic extract/fraction) was also stated in Table 4.6.

From Table 4.6, all of the plant samples (ethanolic extract/fraction) were not active in killing HT-29 cells as the IC_{50} values obtained were higher than 100 μg/mL. *Euodia redlevi* (fruit&flower), *Garcinia atroviridis* (branch), *Garcinia mangostana* (branch), *Garcinia mangostana* (leaf), *Lawsonia inermis* (branch), *Oxalis barrelieri* (root) and *Tetracera indica* (fruit) ethanolic extracts, and 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract were not cytotoxic to HT-29 cells up to 100 μg/mL because the cell viability was 100% when treated at 100 μg/mL. *Anacardium occidentale* (branch), *Cinnamomum zeylanicum* (branch), *Hibiscus tiliaceus* (leaf), *Leptospermum flavescens* (leaf) and *Peltophorum pterocarpum* (leaf) ethanolic extracts were less toxic to HT-29 cells with cell viability ≥80% when treated at highest concentration, 100 μg/mL. Ethanolic extract of *Andrographis paniculata* (leaf) was slightly toxic to HT-29 cells with the cell viability of 57% at 100 μg/mL. On the

other hand, doxorubicin hydrochloride, cisplatin and ethacrynic acid showed highly active cytotoxicity on HT-29 cells. Doxorubicin hydrochloride is the most active cytotoxic agent compared to cisplatin and ethacrynic acid, with IC_{50} value of 0.79 μg/mL. The IC_{50} values of cisplatin and ethacrynic acid were 9.49 and 19 μg/mL respectively.

Table 4.6 : Cytotoxicity effects of samples on HT-29 cell proliferation

Sample	**IC_{50} (mean ± S.D., µg/mL)**	**Percentage of cell viability at 100 µg/mL (%)**
Doxorubicin hydrochloride	0.79 ± 0.05	ND
Cisplatin	9.49 ± 0.13	ND
Ethacrynic acid	19.0 ± 0.26	1
Ethanolic extract		
Anacardium occidentale (branch)	>100	80
Andrographis paniculata (leaf)	>100	57
Cinnamomum zeylanicum (branch)	>100	99
Euodia redlevi (fruit&flower)	>100	100
Garcinia atroviridis (branch)	>100	100
Garcinia mangostana (branch)	>100	100
Garcinia mangostana (leaf)	>100	100
Hibiscus tiliaceus (leaf)	>100	96
Lawsonia inermis (branch)	>100	100
Leptospermum flavescens (leaf)	>100	83
Oxalis barrelieri (root)	>100	100
Peltophorum pterocarpum (leaf)	>100	82
Tetracera indica (fruit)	>100	100
Fraction		
50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract	>100	100

* MeOH= Methanol, ND= not determined. IC_{50} values indicated are the means (± standard deviation, S.D.) of three independent experiments.

4.9 COMBINATION EFFECTS OF PLANT SAMPLES AND DOXORUBICIN HYDROCHLORIDE ON CYTOTOXICITY OF HT-29 CELLS

In order to examine the potentiation effects of plant sample (ethanolic extract/fraction) and ethacrynic acid on doxorubicin hydrochloride cytotoxicity of HT-29, doxorubicin hydrochloride at different concentrations in combination with fixed concentration of plant sample(ethanolic extract/fraction) or ethacrynic acid were tested against HT-29 cells. *in vitro* cytotoxicity effects of the combination against HT-29 cell line were evaluated using MTT assay. Plant sample (ethanolic extract/fraction) concentration was fixed at 50 μg/mL and ethacrynic acid concentration (as a reference) was fixed at 10 μg/mL. The concentration fixed was the concentration at which viability of HT-29 cells is higher than 95%, when treated alone with plant samples (ethanolic extract/fraction) or ethacrynic acid (Appendix IV, Figure 7.40-7.54).

The dose-response graph for each combination (Appendix IV, Figure 7.55-7.69) and Figure 4.8 which showed the comparison of IC_{50} values between groups of combination were generated using Microsoft Excel software. Table 4.7 showed the IC_{50} values of doxorubicin hydrochloride, alone or in combination with plant samples (ethanolic extract/fraction) or ethacrynic acid. Statistical analysis of data obtained was done by using SigmaPlot 12.0 analytical software.

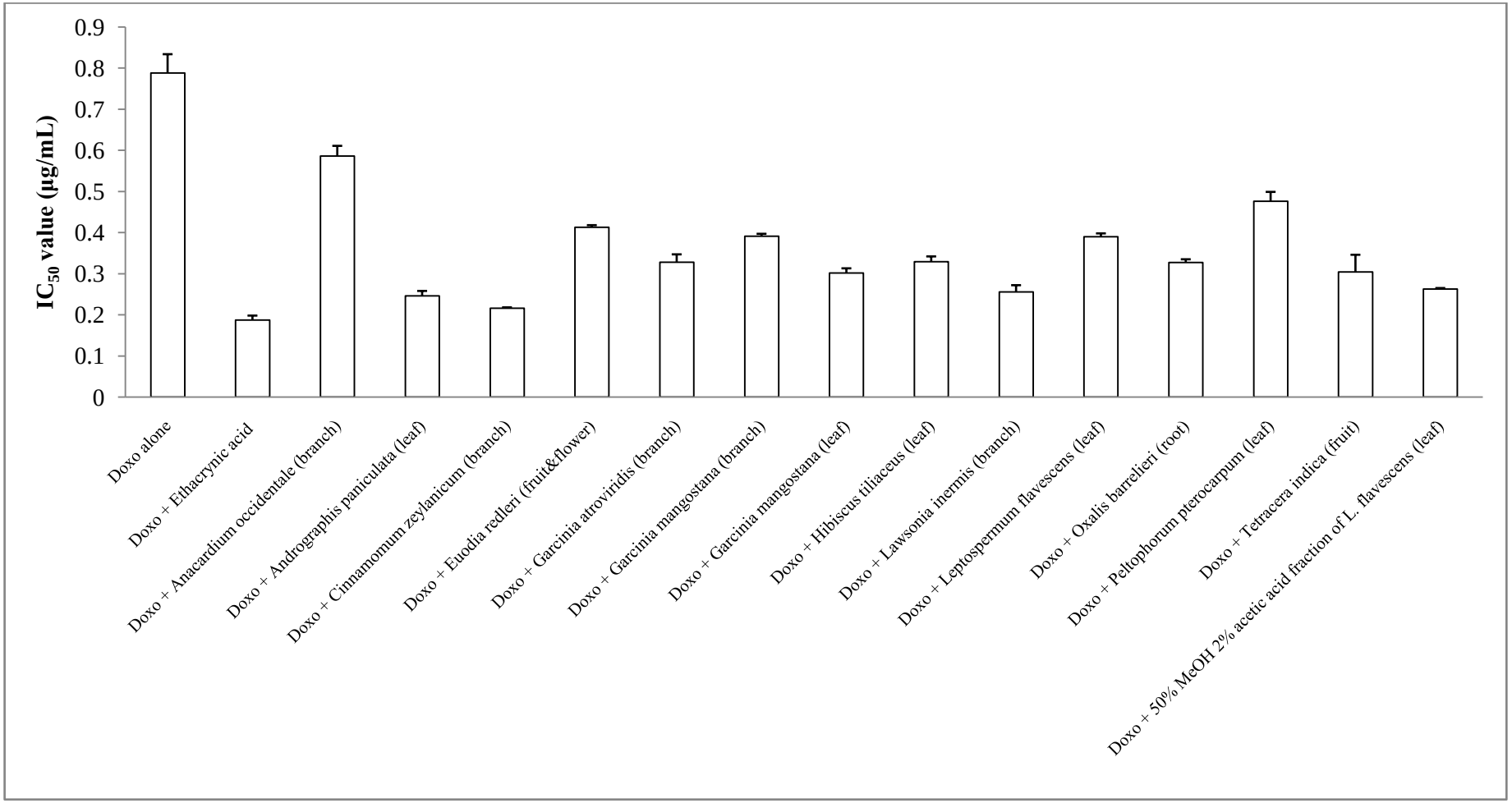

Figure 4.8 : Comparison of IC_{50} values of doxorubicin hydrochloride, alone or in combination, on cytotoxicity of HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations. Comparison of IC_{50} values between groups of combination using ANOVA indicates statistically significant difference with P = <0.001. Doxo= Doxorubicin hydrochloride.

Based on Figure 4.8, all of the plant samples (ethanolic extract/fraction) demonstrated potentiation effect on doxorubicin hydrochloride cytotoxicity of HT-29 cells. The IC_{50} value was significantly ($P < 0.05$) lower for all combinations compared with doxorubicin hydrochloride alone. Ethacrynic acid which is well-known as GST-P inhibitor was used as a reference in this experiment and the combination of Doxo-ethacrynic acid had the lowest IC_{50} value (0.19 ug/mL), followed by Doxo-*Cinnamomum zeylanicum* (branch) (0.22 ug/mL), Doxo-*Andrographis paniculata* (leaf) (0.25 ug/mL), Doxo-*Lawsonia inermis* (branch) (0.26 ug/mL), Doxo-50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) (0.26 ug/mL), Doxo-*Garcinia mangostana* (leaf) (0.30 ug/mL), Doxo-*Tetracera indica* (fruit) (0.30 ug/mL), Doxo-*Garcinia atroviridis* (branch) (0.33 ug/mL), Doxo-*Hibiscus tiliaceus* (leaf) (0.33 ug/mL), Doxo-*Oxalis barrelieri* (root) (0.33 ug/mL), Doxo-*Garcinia mangostana* (branch) (0.39 ug/mL), Doxo-*Leptospermum flavescens* (leaf) (0.39 ug/mL), Doxo-*Euodia redlevi* (fruit&flower) (0.41 ug/mL), Doxo-*Peltophorum pterocarpum* (leaf) (0.48 ug/mL) and Doxo-*Anacardium occidentale* (branch) (0.59 ug/mL) (Table 4.7). When the Doxo-ethacrynic acid combination was statistically compared (ANOVA) with the remaining combinations, the difference was significant ($P < 0.05$) in all cases.

Table 4.7 : Cytotoxicity effects of doxorubicin hydrochloride, alone or in combination, on HT-29 cells

Test sample	**IC_{50} of doxorubucin hydrochloride cytotoxicity on HT-29 (ug/mL)**
Doxorubicin hydrochloride alone	0.79 ± 0.05
In combination with:	
Ethacrynic acid	0.19 ± 0.01
Ethanolic extract	
Anacardium occidentale (branch)	0.59 ± 0.03
Andrographis paniculata (leaf)	0.25 ± 0.01
Cinnamomum zeylanicum (branch)	0.22 ± 0.00
Euodia redlevi (fruit&flower)	0.41 ± 0.01
Garcinia atroviridis (branch)	0.33 ± 0.02
Garcinia mangostana (branch)	0.39 ± 0.01
Garcinia mangostana (leaf)	0.30 ± 0.01
Hibiscus tiliaceus (leaf)	0.33 ± 0.01
Lawsonia inermis (branch)	0.26 ± 0.02
Leptospermum flavescens (leaf)	0.39 ± 0.01
Oxalis barrelieri (root)	0.33 ± 0.01
Peltophorum pterocarpum (leaf)	0.48 ± 0.02
Tetracera indica (fruit)	0.30 ± 0.04
Fraction	
50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract	0.26 ± 0.00

* MeOH= Methanol. IC_{50} values indicated are the means (± standard deviation, S.D.) of three independent experiments. Multiple comparisons versus control group (doxorubicin hydrochloride alone) using Holm-Sidak method indicated an overall significance level equal to 0.05.

4.10 COMBINATION EFFECTS OF PLANT SAMPLES AND CISPLATIN ON CYTOTOXICITY OF HT-29 CELLS

In order to examine the potentiation effects of plant sample (ethanolic extract/fraction) and ethacrynic acid on cisplatin cytotoxicity of HT-29, cisplatin at different concentrations in combination with fixed concentration of plant sample (ethanolic extract/fraction) or ethacrynic acid were tested against HT-29 cells. *in vitro* cytotoxicity effects of the combination against HT-29 cell line were evaluated using MTT assay. Plant sample (ethanolic extract/fraction) concentration was fixed at 50 μg/mL and ethacrynic acid concentration (as a reference) was fixed at 10 μg/mL. The concentration fixed was the concentration at which viability of HT-29 cells is higher than 95%, when treated alone with plant samples (ethanolic extract/fraction) or ethacrynic acid (Appendix IV, Figure 7.40-7.54).

The dose-response graph for each combination (Appendix IV, Figure 7.70-7.84) and Figure 4.9 which showed the comparison of IC_{50} values between groups of combination were generated using Microsoft Excel software. Table 4.8 showed the IC_{50} values of cisplatin, alone or in combination with plant samples (ethanolic extract/fraction) or ethacrynic acid. Statistical analysis of data obtained was done by using SigmaPlot 12.0 analytical software.

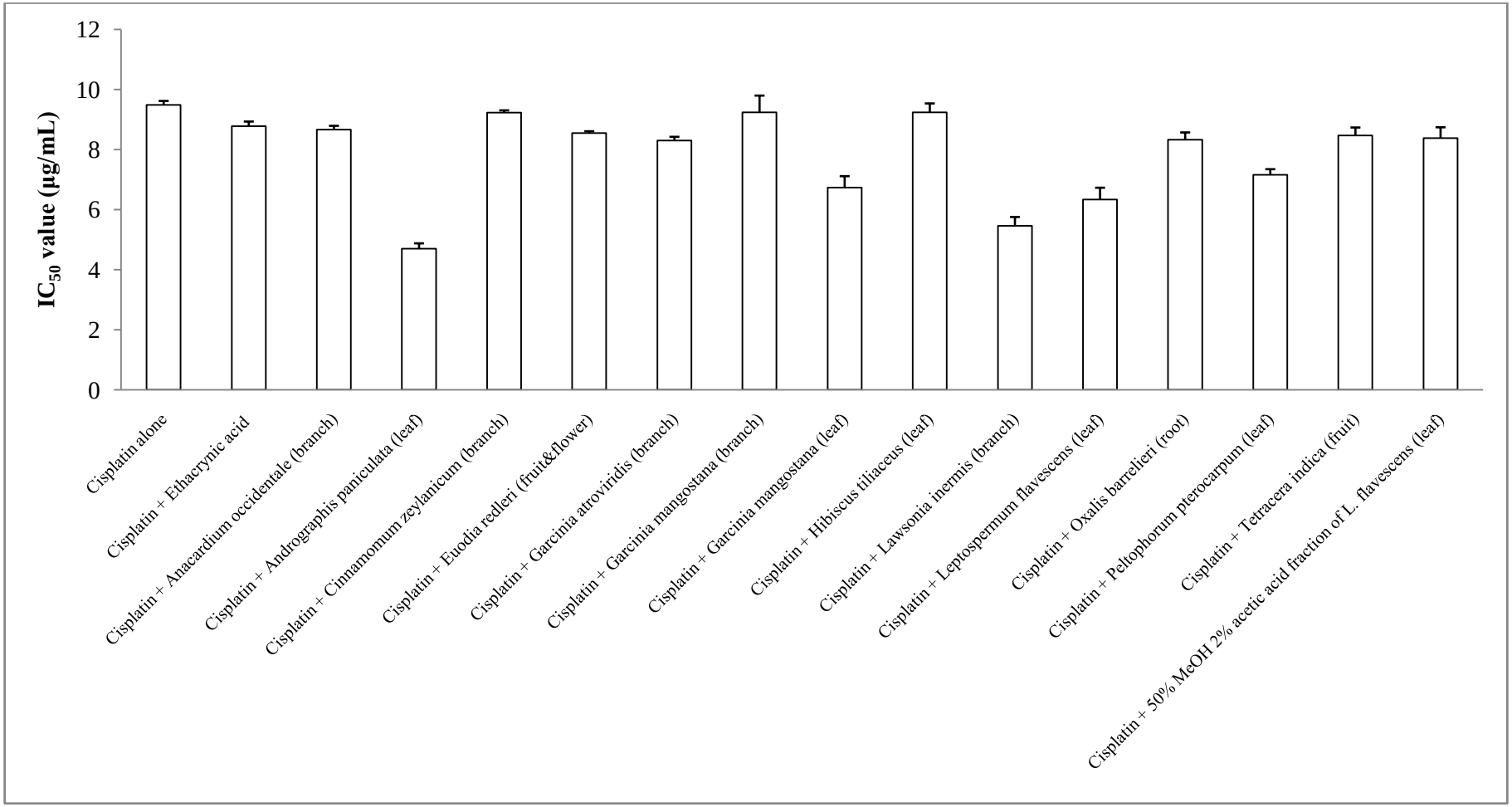

Figure 4.9 : Comparison of IC_{50} values of cisplatin, alone or in combination, on cytotoxicity of HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations. Comparison of IC_{50} values between groups of combination using ANOVA indicates statistically significant difference with P = <0.001.

Figure 4.9 show that all of the plant samples (ethanolic extract/fraction) demonstrated potentiation effect on cisplatin cytotoxicity of HT-29 cells. The IC_{50} value was significantly ($P < 0.05$) lower for all of the combinations compared with cisplatin alone, except for the combinations of Cisplatin-*Cinnamomum zeylanicum* (branch), Cisplatin-*Hibiscus tiliaceus* (leaf) and Cisplatin-*Garcinia mangostana* (branch). Ethacrynic acid which is well-known as GST-P inhibitor was used as a reference in this experiment. However, the combination of Cisplatin-ethacrynic acid does not exhibit lowest IC_{50} value (8.78 ug/mL) compared with the combinations of cisplatin with plant samples (ethanolic extract/fraction). Cisplatin-*Andrographis paniculata* (leaf) was the combination which had the lowest IC_{50} value (4.70 ug/mL), followed by Cisplatin-*Lawsonia inermis* (branch) (5.46 ug/mL), Cisplatin- *Leptospermum flavescens* (leaf) (6.33 ug/mL), Cisplatin-*Garcinia mangostana* (leaf) (6.74 ug/mL), Cisplatin-*Peltophorum pterocarpum* (leaf) (7.16 ug/mL), Cisplatin-*Garcinia atroviridis* (branch) (8.30 ug/mL), Cisplatin-*Oxalis barrelieri* (root) (8.33 ug/mL), Cisplatin-50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) (8.38 ug/mL), Cisplatin-*Tetracera indica* (fruit) (8.47 ug/mL), Cisplatin-*Euodia redlevi* (fruit&flower) (8.55 ug/mL), Cisplatin-*Anacardium occidentale* (branch) (8.66 ug/mL), Cisplatin-*Cinnamomum zeylanicum* (branch) (9.23 ug/mL), Cisplatin-*Garcinia mangostana* (branch) (9.24 ug/mL) and Cisplatin-*Hibiscus tiliaceus* (leaf) (9.24 ug/mL) (Table 4.8). When the combination of Cisplatin-ethacrynic acid was statistically compared (ANOVA) with the other combinations, the difference was significant ($P < 0.05$) for Cisplatin-*Andrographis paniculata* (leaf), Cisplatin-*Lawsonia inermis* (branch), Cisplatin- *Leptospermum flavescens* (leaf), Cisplatin-*Garcinia mangostana* (leaf) and Cisplatin-*Peltophorum pterocarpum* (leaf), but not for the others.

Table 4.8 : Cytotoxicity effects of cisplatin, alone or in combination, on HT-29 cells

Test sample	**IC_{50} of cisplatin cytotoxicity on HT-29 (µg/mL)**
Cisplatin alone	9.49 ± 0.13
In combination with:	
Ethacrynic acid	8.78 ± 0.15
Ethanolic extract	
Anacardium occidentale (branch)	8.66 ± 0.13
Andrographis paniculata (leaf)	4.70 ± 0.18
Cinnamomum zeylanicum (branch)	9.23 ± 0.07
Euodia redlevi (fruit&flower)	8.55 ± 0.06
Garcinia atroviridis (branch)	8.30 ± 0.12
Garcinia mangostana (branch)	9.24 ± 0.55
Garcinia mangostana (leaf)	6.74 ± 0.38
Hibiscus tiliaceus (leaf)	9.24 ± 0.30
Lawsonia inermis (branch)	5.46 ± 0.30
Leptospermum flavescens (leaf)	6.33 ± 0.40
Oxalis barrelieri (root)	8.33 ± 0.24
Peltophorum pterocarpum (leaf)	7.16 ± 0.19
Tetracera indica (fruit)	8.47 ± 0.26
Fraction	
50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract	8.38 ± 0.36

* MeOH= Methanol. IC_{50} values indicated are the means (± standard deviation, S.D.) of three independent experiments. Multiple comparisons versus control group (cisplatin alone) using Holm-Sidak method indicated an overall significance level equal to 0.05.

5.0 DISCUSSION

5.1 PLANT EXTRACTION

The application of natural resources in primary health care has become more and more important especially in developing countries. In recent years, there is a revival of interest in drug discovery from natural products including medicinal plants. Numbers of pharmacognostical and pharmacological investigations are undergoing to identify potential drugs or structural models for the design and development of novel therapeutic drugs used in the treatment of diseases such as cancer and infectious disease. Analysis of the origin of newly identified therapeutic drugs has revealed a significant role of natural products in discovery and development of medication drugs (Newman *et al*., 2003).

In present study, the plant extraction was done according to the fractionation protocol introduced by Claeson *et al.* (1998). This polypeptide isolation protocol was selected because our study is dedicated to extract bioactive peptides from local plants. Furthermore, the fractionation procedures removed ubiquitous compounds known to interfere with bioassays often used in pharmacological investigations. Peptide inhibitors were targeted in this study because many peptides have been discovered as therapeutic pharmaceutical drugs, vasodilators, hormones and neuropeptides (da Rocha Pitta *et al*., 2010; Daly *et al*., 2012; Reglodi *et al*., 2011; Lico *et al*., 2012).

43 local plant samples (31 species with different plant parts) selected in this study were pre-extracted with dichloromethane, followed by aqueous alcohol extraction with 50% ethanol and lyophilized. Pre-extraction with dichloromethane removed lipophilic substances such as lipids, chlorophyll and low molecular weight compounds (example, terpenoids). Since polypeptides are insoluble in dichloromethane, it will remain in the plant residue and thus extraction with 50% ethanol had extracted most of

the polypeptides of the plant sample. Polypeptides were extracted with 50% ethanol instead of pure water or alcohol due to better solubility of polypeptides and preservative properties of aqueous alcohol. In addition, polysaccharides and enzymes are removed from the ethanolic extract since these compounds are insoluble in 50% ethanol (Claeson *et al*., 1998). Plant ethanolic extracts were then lyophilized and stored at 4℃ because lyophilization enhanced stability of sample in dry state and removed water without excessive heating which may affect the bioactivities of sample (Food and Drug Administration, 2009).

5.2 HT-29 CELL LINE

HT-29 cell line is one of the best described cell line among several adenocarcinoma cell lines and has been extensively used in pharmacological study. This cell line was derived from human primary colon adenocarcinoma (von Kleist *et al*., 1975). From Figure 4.3, we can see that the cells have epitheloid morphology and cells were roughly spherical. Some of the ultrastructural features of HT-29 cells described by ATCC (2013) were captured in Figure 4.3, such as large vacuolated mitochondria with dark granules, lipid droplets and lysosomes. The cells were grown in monolayer which attached to the surface of the culture flask.

HT-29 cell line was often used in the studies related to GST inhibition, especially for GST-P (Ciaccio *et al*., 1995; Tashiro *et* al., 2001; Shen *et al*., 2003). High expression of GST-P and multidrug resistance nature of HT-29 cell line made it the first choice in clonogenic assays related to drug potentiation through GST inhibition (Morgan *et al*., 1996; Tew *et al*., 1988). GST-P was targeted as a model for the synthesis of specific inhibitor which could be used as adjuvant in chemotherapy (Oakley *et al*., 1997).

5.3 GST-P PURIFICATION AND IDENTIFICATION

GST-P was purified and identified with a combination of affinity chromatography, enzyme assays, SDS-PAGE and IEF analysis, and ionization mass spectrometry of MALDI-TOF. GST-P was isolated from the HT-29 cell lysate by affinity chromatography. Catalytic activity of GST towards CDNB was measured for unbound fraction and GST eluate (result no shown). GST activity is only detectable in GST eluate. GST eluate was then concentrated with protein concentrator with the purpose of remove excessive buffer and GSH in the eluate. Specific activity of the isolated GST-P was 0.032 μmol/min/mg. Based on the result of peptide mass fingerprint analysis (Appendix IV), the identified nominal mass and pI value of isolated GST-P were 23.3 kDa and 5.4 respectively. The mass obtained was slightly different from the mass determined by SDS-PAGE (23.8 kDa). This is probably due to the difference in separating parameter of the two methods in which SDS-PAGE separated proteins by their size while MALDI-TOF separated the proteins by molecular mass. The pI value obtained from IEF analysis (Section 4.3.4) was matched with the pI value obtained from MALDI-TOF (Appendix IV). Single band on IEF gel (Figure 4.6) indicates that there is only one isoform of GST-P isolated from HT-29 cell line.

Since there is only GST-P isolated in this investigation, the finding is in agreement with those of Beaumont *et al*. (1998) who reported that only GST-P was expressed in HT-29 cells but not for alpha or mu-class of GST, and Schultz *et al*. (1997) who showed that only mRNA of GST-P quantified from HT-29 cell line.

5.4 PRELIMINARY SCREENING ON GST-P INHIBITION

A total of 43 plant ethanolic extracts which extracted from 31 local plant species (with different plant parts) were screened for their inhibitory effects on GST-P. To our knowledge, this is the first report of detailed study in *in vitro* inhibition of human GST-P by local plant extracts. 30 ethanolic extracts showed inhibitory effects on GST-P with relative changes in the range of 4-100% reduction (Table 4.1). The remaining 13 ethanolic extracts increased activity of GST-P. Ethanolic extracts which enhanced the activity of GST-P were not continued with downstream experiments due to the reason that GST-P activator is not expected for this experiment. However, the finding of the GST activator in plant has implications for developing functional foods to boost up body immune system and also food-related precautions during chemotherapy to prevent drug resistance contributed by activation of GST-P.

Among the 30 ethanolic extracts which have inhibitory effects on GST-P, only ethanolic extracts with GST-P inhibition percentage higher than 50% were selected for downstream. This is because of the criteria for reporting IC_{50} which require maximum inhibition percentage to be greater than 50% (National Institutes of Health, 2010). The selected samples include *Anacardium occidentale* (branch), *Andrographis paniculata* (leaf), *Cinnamomum zeylanicum* (branch), *Euodia redlevi* (fruit&flower), *Garcinia atroviridis* (branch), *Garcinia mangostana* (branch and leaf), *Hibiscus tiliaceus* (leaf), *Lawsonia inermis* (branch), *Leptospermum flavescens* (leaf), *Oxalis barrelieri* (root), *Peltophorum pterocarpum* (leaf) and *Tetracera indica* (fruit) ethanolic extracts.

Results in Table 4.1 demonstrated different parts of the same plant could exert distinct effects on GST-P. For example, branch and fruit of *Piper nigrum*. *Piper nigrum* (branch) ethanolic extract inhibited 21% of GST-P activity while *Piper nigrum* (fruit) ethanolic extract induced 97% of GST-P activity. A possible explanation for this might

be the variation in chemical constituents of different plant parts (Padalia *et al*., 2012; Das *et al*., 2002).

5.5 BIOASSAY-GUIDED FRACTIONATION

Based on the results of preliminary screening on GST-P inhibition by plant ethanolic extracts (Table 4.1), *Garcinia atroviridis* (branch) and *Leptospermum flavescens* (leaf) ethanolic extracts has a 100% inhibition on GST-P activity and thus proceeded with bioassay-guided fractionation using polyamide chromatography. Undesired polyphenolic compounds of plant ethanolic extract, such as tannins, were removed when passed through polyamide column. Polyphenolic compounds were irreversibly bound to polyamide and thus retained in the column. Peptides were not bind to polyamide and thus eluted with 2% acetic acid. 2% acetic acid was used as eluting solvent due to the facts that lower pH increases binding affinity of polyamide towards tannins, increases solubility of proteins and reduces effects of proteases (Claeson *et al*., 1998; Ohishi *et al*., 2003). However, there were certain peptides insoluble in 2% acetic acid and these peptides were then eluted with 50% methanol 2% acetic acid. Proteins insoluble in 2% acetic acid are protein with high surface hydrophobicity (Okada *et al*., 1988).

Based on the results in Table 4.2, we found that only the 50% methanol 2% acetic acid fractions of *Garcinia atroviridis* (branch) and *Leptospermum flavescens* (leaf) ethanolic extracts demonstrated inhibition activity on GST-P. It seems possible that the acetic acid-insoluble peptides present in 50% methanol 2% acetic acid fraction have higher surface hydrophobicity (Okada *et al*., 1988). This is also consistent with those of other studies suggested that GST-P tends to bind with hydrophobic substances at the ligandin site of the enzyme. The GSH and ligand binding sites of GST are located at

different parts of the same active site cleft, and the ligand site is located in the electrophile binding site (Oakley *et al*., 1997, 1999; Vararattanavech *et al*., 2006). Fractions of *Garcinia atroviridis* (branch) ethanolic extract lose their inhibitory activity after polyamide fractionation and this inconsistency may be due to the reason that the inhibitory agents in ethanolic extract of *Garcinia atroviridis* (branch) inhibited GST-P synergistically instead of individually.

From Table 4.2, we also found that only 50% methanol 2% acetic acid fraction of *Leptospermum flavescens* (leaf) ethanolic extract retained its high inhibition on GST-P activity. Then, we tried to further fractionate this sample in order to isolate the pure form of the inhibitor. Firstly, we injected the 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract into a C_{18} reversed-phase choromatography (RPC) column and ran with 0.12% Trifluoroacetic acid (TFA). The peptides were eluted in gradients with 70% methanol: 0.1% TFA. The fractions obtained were then subjected to GST-P inhibitory assay. Unfortunately, there was no inhibitory activity found for all the fractions tested. Then, we tried with a series of hydrophobic interaction chromatography (HIC) columns consist of Phenyl Sepharose, Butyl Sepharose, Butyl-S Sepharose and Octyl Sepharose. The running buffer used was 50 mM sodium phosphate, 1 M ammonium sulphate, pH 7.0, and the eluting buffer was 50 mM sodium phosphate, pH 7.0 (as recommended by manufacturer). The fractions collected were subjected to GST-P inhibitory activity assays and the results obtained (no shown) were either less or no reduction in GST-P activity. After that, we switched to another column, which is Superdex G-75 gel filtration chromatography column. The running buffer used was 50 mM sodium phosphate, pH 7.0. The GST-P inhibitory effects of fractions collected was also found to be very low.

Despite repeated attempts to separate the mixture by different types of chromatography, it does not prove possible to separate the components in 50%

methanol 2% acetic acid fraction of *Leptospermum flavescens* (leaf) ethanolic extract. We assumed that probably the peptides were degraded during the process of isolation or there might be synergy effects of different peptides on inhibition of GST-P, which will be diminish if the peptides exist in pure form. The synergism may also appear as interaction of more than one agent which results a combined effect greater than the sum of individual parts (that is addictive effect) (Pengelly, 2004).

5.6 IC_{50} OF GST-P INHIBITORY ASSAYS

IC_{50} value is the half maximal inhibitory concentration of inhibitor. It is a quantitative measurement of the effectiveness of a compound in inhibiting an enzymatic reaction at a fixed concentration of substrate (Copeland, 2000). The IC_{50} values of plant samples were determined from dose-response curve relating the concentration of plant sample (ethanolic extract/fraction) to the percentage of inhibition on GST activity, at fixed concentration of GST-P and substrates (GSH and CDNB) (Appendix IV).

A total of 14 plant samples (ethanolic extract/fraction) were tested in this experiment. All of the plant samples (ethanolic extract/fraction) were significantly ($P < 0.5$) inhibited GST-P in dose-dependent manner (Appendix IV). *Cinnamomum zeylanicum* (branch) is the most active sample with IC_{50} value of 0.07 mg/mL, followed by ethanolic extracts of *Leptospermum flavescens* (leaf), *Hibiscus tiliaceus* (leaf), *Garcinia mangostana* (leaf), *Garcinia mangostana* (branch) *Tetracera indica* (fruit), *Oxalis barrelieri* (root), *Garcinia atroviridis* (branch), *Anacardium occidentale* (branch), *Euodia redlevi* (fruit&flower), 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf), *Peltophorum pterocarpum* (leaf), *Andrographis paniculata* (leaf) and *Lawsonia inermis* (branch) ethanolic extracts which have IC_{50} values of 0.09-0.46 mg/mL. Compared to previous reported plant extract inhibition activity on GST, some of our

ethanolic extracts have higher potency. Çoruh *et al*. (2007) reported that extracts of *Gundelia tournefortii* L. aerial and seeds have IC_{50} values of 0.155 and 0.097 mg/mL respectively. Pr ńcipe and Spira (2009) described GST inhibitory effects of *Bauhinia forficate*, *Jacaranda puberula* and *Pimenta pseudocaryophyllus* extracts, which IC_{50} values are 0.1-1, > 0.1 and > 0.1 mg/mL respectively.

The results obtained in this experiment (Section 4.5) were not consistent with previous screening results (section 4.4). There are several possible explanations for the results, such as (1) samples are in mixture and probably there are presence of compounds (for example, tannins and pigments) which might interfere with the bioassays (Claeson *et al*., 1998), (2) samples have steep dose-response curves that different from stoichiometric inhibition which predicts that changes of IC_{50} should be linear with enzyme concentration, (3) more than one type of inhibitors exist in the sample mixture, (4) physical phase transition of inhibitory agent and (5) enzyme concentration is greater than the dissociation constant (K_d) of inhibitor (Shoichet, 2006).

5.7 KINETIC STUDIES

Enzyme kinetics is the study of enzyme catalysis reactions. Measurement and mathematical description of rate of reaction and its associated constants are investigated in enzyme kinetics study (Rogers and Gibon, 2009). In this study, inhibitory kinetics of plant samples (ethanolic extract/fraction) on the V_{max} and K_m values of GST-P were determined. V_{max} and K_m values of GST-P with or without binding of inhibitor were determined by Michaelis-Menten equation.

Based on the results in Table 4.4, *Anacardium occidentale* (branch), *Cinnamomum zeylanicum* (branch), *Euodia redlevi* (fruit&flower), *Garcinia atroviridis* (branch), *Garcinia mangostana* (branch), *Hibiscus tiliaceus* (leaf), *Leptospermum*

flavescens (leaf), *Oxalis barrelieri* (root), *Peltophorum pterocarpum* (leaf), *Tetracera indica* (fruit) ethanolic extracts and 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract were displayed mixed mode inhibition on GST-P with reduced V_{max} and increased K_m of GST-P. Increased K_m was due to the interference of inhibitors with substrate binding while reduced V_{max} owed to hindered catalysis of enzyme-substrate complex (Segel, 1993). The mechanism of mixed mode inhibition comprised of competitive, noncompetitive and uncompetitive inhibition (Rogers and Gibon, 2009). Findings of mixed mode inhibition further support the idea of more than one type of inhibitors exist in the sample mixture, different inhibitors exerted different types of inhibition on GST-P. Several other plant extracts were reported to exhibit mixed type of inhibition on enzyme, for instance, *Theobroma cacao* extract which inhibited pancreatic lipase in mixed mode (Gu, *et al*., 2011) and *Olea europaea* L. leaf extract which displayed mixed mode inhibition on xanthine oxidase (Flemmig, 2011). Mixed inhibition of *Anacardium occidentale* (branch), *Euodia redlevi* (fruit&flower), *Garcinia atroviridis* (branch), *Peltophorum pterocarpum* (leaf) ethanolic extracts and 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on GST-P greatly reduced the substrate binding affinity of GST-P (high K_m values, ≥ 3.0 mM).

On the other hand, *Andrographis paniculata* (leaf), *Garcinia mangostana* (leaf) and *Lawsonia inermis* (branch) ethanolic extracts uncompetitively inhibited GST-P with respect to varied GSH concentration (Table 4.4). Uncompetitive inhibitors in these ethanolic extracts bind to the enzyme-substrate complex thus lowering the K_m and V_{max} of GST-P. Lowered K_m and V_{max} were due to the reduction in effective concentration of enzyme–substrate complex which leads to the increases in enzyme's affinity for its substrate and decreases in maximum activity of enzyme (Copeland, 2000). Previously reported plant extracts which have uncompetitive inhibitory activities include aerial extracts of *Citrullus colocynthis* and *Ipomoea pescaprae* which uncompetitively

inhibited alpha-glucosidase (Gurudeeban *et al*., 2012), extract of *Manilkara indica* containing Isoaffinetin which is an uncompetitive inhibitor of aldose reductase (Haraguchi *et al*., 2003) and aqueous stem bark extract of *Adenium obesum* which demonstrated uncompetitive inhibition on acetyl cholinesterase activity (Mgbojikwe, 2000).

5.8 CYTOTOXICITY EFFECTS OF SAMPLES ON MRC-5 AND HT-29 CELLS

MRC-5 cell line derived from fetal lung tissue was first introduced by Jacobs *et al.* (1970). The human normal cell line, MRC-5, was initially established in the development of vaccines (Chonmaitree *et al*., 1988; Gregory and Menegus, 1983; Shinohara *et al*., 2002), but later on it was also widely used in biocompatibility assays for pharmacological investigations (Grare *et al*., 2007; Malek *et al*., 2009). In this study, MRC-5 cell line was used in biocompatibility testing of plant samples (ethanolic extract/fraction) cytotoxicity on normal cells. MRC-5 was selected because the cell line has rapid growth rate, longer life span, normal characteristics with no neoplatic properties and tolerance to environmental changes (Jacobs *et al*., 1970; Friedman and Koropchak, 1978).

From the results obtained in section 4.7 and 4.8, all of the plant samples (ethanolic extract/fraction) tested was not toxic to both MRC-5 and HT-29 cell lines at concentration up to 50 μg/mL. That mean, with the presence of the plant sample (ethanolic extract/fraction) alone will not trigger apoptosis in the cells at concentration below 50 μg/mL. Besides, all of the plant samples (ethanolic extract/fraction) have IC_{50} values higher than 100 μg/mL for both HT-29 and MRC-5 cells and according to the selection criteria of American National Cancer Institute (NCI), only crude extract with IC_{50} lower than 30 mg/mL promising the presence of cytotoxic agents (Suffness and

Pezzuto, 1990). At the concentration of 100 μg/mL, *Garcinia mangostana* (branch), *Lawsonia inermis* (branch) and *Tetracera indica* (fruit) ethanolic extracts have minor cytotoxic effect on MRC-5 with up to 35% cell killed. On the other hand, *Andrographis paniculata* (leaf) ethanolic extract was slightly toxic to HT-29 cells, with 43% cell killed at concentration of 100 μg/mL. The reason for this is not clear but it might be related to the activation of apoptotic pathways with increased concentration of the plant sample (ethanolic extract/fraction).

There were three commercial drugs tested in this investigation that is doxorubicin hydrochloride, cisplatin and ethacrynic acid. Doxorubicin and cisplatin are common chemotherapy drugs used in cancer treatment while ethacrynic acid is a well-known GST-P inhibitor. Among the three drugs, doxorubicin hydrochloride was most cyotoxic to both MRC-5 and HT-29 cells with IC_{50} values of 0.82 and 0.79 μg/mL respectively, followed by cisplatin (IC_{50}= 4.07 μg/mL for MRC-5, 9.49 μg/mL for HT-29) and ethacrynic acid (IC_{50}= 22.71 μg/mL for MRC-5, 19.0 μg/mL for HT-29). Based on the IC_{50} values obtained, doxorubicin hydrochloride and ethacrynic acid have higher toxicity effect on HT-29 cells compared to MRC-5 cells while cisplatin more toxic to MRC-5 cells compared to HT-29 cells. The results obtained were consistent with those reported by Malek *et al*. (2011) and Park *et al*. (2012), but not for ethacrynic acid which is not reported before. Ehtacrynic acid was later on used as reference compound in combination assays for its known inhibition effect on GST-P.

5.9 COMBINATION EFFECTS OF PLANT SAMPLES AND DOXORUBICIN HYDROCHLORIDE ON CYTOTOXICITY OF HT-29 CELLS

Doxorubicin is a type of anthacycline antibiotic commonly used in cancer chemotherapy. It has demonstrated significant antitumor activity against different types of cancer in various in vitro and in vivo human tumor xenograft models and clinical trials (Kratz *et* al., 1998; Wendel *et al*., 2004; Kattan *et al*., 1992). Nevertheless, life-threatening illness of neutropenia is the dose-limiting toxicity of doxorubicin. Combination treatment of doxorubicin with other agents was suggested to palliate the drug toxicity which is an important consideration (Seiden *et al*., 2002).

In this experiment, HT-29 cells were treated with doxorubicin hydrochloride in combination with non-cytotoxic doses of plant samples (ethanolic extract/fraction) in order to see the potentiation effects of plant samples (ethanolic extract/fraction) on doxorubicin cytotoxicity of HT-29. Since the concentration of plant sample (50 μg/mL) used in combination assays showed no cytotoxic effect on HT-29, the cytotoxicity on HT-29 resulted by the treatment of combinations of doxorubicin hydrochloride and plant samples (ethanolic extract/fraction) were solely dependent on the concentration of doxorubicin hydrochloride.

By referring to Figure 4.9, we can see that all of the plant samples (ethanolic extract/fraction) and ethacrynic acid (reference compound) potentiated cytotoxicity effects of doxorubicin hydrochloride on HT-29 cells. The combination of doxo-ethacrynic acid has the lowest IC_{50} value of 0.19 μg/mL. Ethacrynic acid at non-cytotoxic concentration (10 μg/mL) has resulted a substantial increase in cytotoxic activity of doxorubicin hydrochloride by 76% reduction in IC_{50} value (from 0.79 μg/mL to 0.19 μg/mL). Among the combinations of doxorubicin hydrochloride and plant samples (ethanolic extract/fraction), combination of doxo-*Cinnamomum zeylanicum*

(branch) has the lowest IC_{50} value with IC_{50}= 0.22 μg/mL. This is corroborated by the findings in GST-P inhibitory assays which showed that *Cinnamomum zeylanicum* (branch) ethanolic extract is the most active extract inhibitor of GST-P. The strength of enhancement in doxorubicin hydrochloride cytotoxicity by plant samples (ethanolic extract/fraction) with respect to reduction of IC_{50} values are such as *Cinnamomum zeylanicum* (branch) > *Andrographis paniculata* (leaf) > *Lawsonia inermis* (branch) > 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) > *Garcinia mangostana* (leaf) ≥ *Tetracera indica* (fruit) > *Oxalis barrelieri* (root) ≥ *Garcinia atroviridis* (branch) ≥ *Hibiscus tiliaceus* (leaf) > *Leptospermum flavescens* (leaf) ≥ *Garcinia mangostana* (branch) > *Euodia redlevi* (fruit&flower) > *Peltophorum pterocarpum* (leaf) > *Anacardium occidentale* (branch).

Thus, with GST-P inhibition by plant samples (ethanolic extract/fraction) and ethacrynic acid and significant ($P < 0.05$) enhancement of doxorubicin hydrochloride cytotoxicity on HT-29 cells by combinations of doxorubicin hydrochloride with either plant samples (ethanolic extract/fraction) or ethacrynic acid, it is possible that inhibition of GST-P potentiated doxorubicin cytotoxicity in HT-29 cells.

Although the results obtained was differ from the finding of Beaumont *et al*. (1998) who showed that neither ethacrynic acid nor other GST inhibitors able to potentiate antitumor effect of doxorubicin, they are consistent with those of Nagourney *et al*. (1990), Tew *et al*. (1988) and Maeda (1993) who demonstrated enhancement on antitumor activity of antineoplatic drug by GST inhibition. Hence, our findings further support the idea of inhibition on GST-P able to enhance the anticancer effects of doxorubicin. Indeed, GST-P may play a role in multidrug resistance mechanism of doxorubicin-resistant cancer cells.

5.10 COMBINATION EFFECTS OF PLANT SAMPLES AND CISPLATIN ON CYTOTOXICITY OF HT-29 CELLS

Cisplatin is a type of platinum-based chemotherapy drug used in the treatment of various cancers for over three decades. The initial respond of treatment with cisplatin is usually high but most of the patient developed relapse after a certain period of treatment due to the development of cisplatin resistance (Stordal and Davey, 2007; Gerl *et al*., 1997). The mechanisms of cisplatin resistance proposed by Rabik and Dolan (2007) include reduced uptake and increased efflux of the drug, detoxification of the drug, DNA tolerance and recovery. So, combination of treatment of cisplatin and agent which able to sensitize the cancer cells to the drug will lead to improvements in the treatment of many cancers.

In this experiment, HT-29 cells were treated with cisplatin in combination with non-cytotoxic doses of plant samples (ethanolic extract/fraction) in order to see the sensitization effects of plant samples (ethanolic extract/fraction) on cisplatin cytotoxicity of HT-29. Non-cytotoxic doses of plant samples (50 μg/mL) used in combination assays are independent factor in the determination of cytotoxic effect of combination of cisplatin with plant sample (ethanolic extract/fraction) on HT-29 cell line. Hence, the cytotoxicity on HT-29 was solely dependent on the concentration of cisplatin.

The cytotoxic effect of combination of ethacrynic acid and cisplatin (IC_{50} = 8.78 μg/mL) on HT-29 cells was not much different from the effect of cisplatin alone (IC_{50} = 9.49 μg/mL). The possible explanation is the cells were resistant to cisplatin which may be due to the reasons that (1) induction of GST-P expression by ethacrynic acid enhanced resistance of cells to cisplatin and (2) increase in expression of other detoxifying agents such as dihydrodiol dehydrogenase (DDH) and gamma-

glutamylcysteine synthetase by alteration of intraclellular thiol status or generation of oxidative stress resulted from exposure of cells to ethacrynic acid (Ciaccio *et al*., 1995). Since cisplatin resistance is multifactorial and to overcome the resistance may depends on the specific inhibition on particular pathway (Stordal and Davey, 2007), yet inhibition on GST-P had no sensitizing effect on cytotoxicity of cisplatin, suggesting that the inhibition of GST-P was not directly involve in cisplatin resistance.

Based on results in Table 4.8, *Anacardium occidentale* (branch), *Cinnamomum zeylanicum* (branch), *Euodia redlevi* (fruit&flower), *Garcinia atroviridis* (branch), *Garcinia mangostana* (branch), *Hibiscus tiliaceus* (leaf), *Oxalis barrelieri* (root), *Tetracera indica* (fruit) ethanolic extracts and 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract showed no or less enhancement on cisplatin cytotoxicity in HT-29 cells, with ≤ 13% reduction of IC_{50} values of cisplatin. *Garcinia mangostana* (leaf), *Leptospermum flavescens* (leaf) and *Peltophorum pterocarpum* (leaf) ethanolic extracts have little enhancement little enhancement on cisplatin cytotoxicity in HT-29 cells, with 29%, 33% and 25% of reduction in IC_{50} values of cisplatin respectively. The enhancement by these ethanolic extracts might be due to mechanisms other than GST-P inhibition. However, ethanolic extracts of *Andrographis paniculata* (leaf) and *Lawsonia inermis* (branch) incredibly increased the cytotoxicity of cisplatin on HT-29 cells. This is probably caused by the inhibition of not only GST-P but also for other signaling pathways (such as multidrug resistance and apoptosis) which induced apoptosis in HT-29 cells. Besides, combinations of cisplatin with either *Andrographis paniculata* (leaf) or *Lawsonia inermis* (branch) ethanolic extracts might be more effective in inhibiting GST activity than plant sample (ethanolic extract/fraction) alone (synergism effect). Hence, lowered application dosage of cisplatin might be able to reduce its cytotoxicity to normal cells.

6.0 CONCLUSION

In conclusion, the results of this study showed that local plants have potent inhibitors of GST pi which able to overcome the drug resistance of cancerous cells. Based on the results of preliminary GST-P inhibition screening, 30 ethanolic extracts displayed inhibition activity on GST-P and 13 out of the 30 ethanolic extracts have inhibition effects higher than 50%. *Garcinia atroviridis* (branch) and *Leptospermum flavescens* (leaf) ethanolic extracts have the highest inhibitory effect with a 100% inhibition on GST pi activity. The 13 ethanolic extracts were subjected for downstream experiments consist of IC_{50} determination, kinetic studies and cytotoxicity assays. Results of IC_{50} determination assay suggested that the most active GST-P inhibiting sample was *Cinnamomum zeylanicum* (branch) ethanolic extract with IC_{50} value of 0.07 mg/mL, followed by *Leptospermum flavescens* (leaf) and *Hibiscus tiliaceus* (leaf) ethanolic extracts with IC_{50} values of 0.09 and 0.10 mg/mL respectively. Meanwhile for the kinetic analysis, all of the ethanolic extracts shown mixed mode inhibition on GST-P activity, except for the ethanolic extracts of *Andrographis paniculata* (leaf), *Garcinia mangostana* (leaf) and *Lawsonia inermis* (branch) which uncompetitively inhibited GST-P. All of the ethanolic extracts were showed to be non toxic to both MRC-5 and HT-29 cell lines, with IC_{50} value >100 μg/mL. Combination studies indicated that GST-P inhibition able to potentiate the cytotoxicity of doxorubicin hydrochloride on HT-29 cells, but not for cisplatin. Combination of doxorubicin hydrochloride-*Cinnamomum zeylanicum* (branch) ethanolic extract has the lowest IC_{50} value with IC_{50}= 0.22 μg/mL. Nevertheless, *Andrographis paniculata* (leaf) and *Lawsonia inermis* (branch) ethanolic extracts were incredibly increase the cytotoxicity of cisplatin on HT-29 cells with IC_{50} values of 4.70 and 5.46 μg/mL respectively. On the other hand, bioassay-guided fractionation of *Leptospermum flavescens* (leaf) ethanolic extract obtained a fraction of 50% methanol 2% acetic acid which has 95% inhibition on GST-P activity. 50%

methanol 2% acetic acid fraction of *L. flavescens* (leaf) extract inhibited GST-P in mixed mode with IC_{50} value of 0.19 mg/mL. This fraction was not toxic to both HT-29 and MRC-5 cells with IC_{50} value >100 μg/mL. Combination of doxorubicin hydrochloride with 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) extract enhanced doxorubicin hydrochloride cytotoxicity on HT-29 cells with IC_{50}= 0.26 μg/mL whereas combination of cisplatin-50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) extract gave an IC_{50} value of 8.38 μg/mL on cytotoxicity of HT-29 cells. Our results show that local plants can be source of GST-P inhibitors which able to enhance cytotoxicity of anticancer drugs on tumor cells.

Following the investigations described in this dissertation, the future work could be involving extensive strategy of the isolation and structural determination of the inhibitor molecules and thus refine the kinetic evaluation of its behavior towards GST-P.

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APPENDICES

APPENDIX I: AUTHENTICATION OF PLANT SAMPLES

Table 7.1 : Plant authentication

Plant sample	Authenticated by	Place of collection	Parts used (reference number)
Anacardium occidentale (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (AO1); fruit & flower (AO2)
Andrographis paniculata (Wallich, 1832)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (AP1); leaf (AP2)
Artocarpus heterophyllus (Lamarck and Poiret, 1789)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Leaf (AH1)
Averrhoa bilimbi (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (AB1); leaf (AB2)
Cinnamomum zeylanicum (Blume, 1826a)	Prof. Dr. Ong Hean Chooi	Jabatan Kejuruteraan, Universiti Malaya	Branch (CZ1)
Commelina nudiflora (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Ladang ISB, Universiti Malaya	Whole plant (CN1)
Euodia redlevi (David, 1995)	Prof. Dr. Ong Hean Chooi	Kebun ISB, Universiti Malaya	Flower & fruit (ER1)
Eupatorium odoratum (Linnaeus, 1759)	Prof. Dr. Ong Hean Chooi	Gombak, Kuala Lumpur	Branch (EO1)
Fagraea fragrans (Roxburgh, 1824)	Prof. Dr. Ong Hean Chooi	Jabatan Pergigian, Universiti Malaya	Leaf & branch (FF1); fruit & flower (FF2)
Ficus auriculata (Loureiro, 1790)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Leaf (FA1)
Garcinia atroviridis (Hooker, 1875)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (GA1)
Garcinia mangostana (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (GM1); leaf (GM2)
Hibiscus tiliaceus (Linnaeus, 1753b)	Prof. Dr. Ong Hean Chooi	Kerinchi, Kuala Lumpur	Leaf (HT1)

Table 7.1, continued

Plant sample	Authenticated by	Place of collection	Parts used (reference number)
Ipomoea aquatica (Forsskål, 1775)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Leaf (IA1)
Justicia gendarussa (Burman, 1768)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (JG1)
Lagerstroemia speciosa (Persoon, 1806)	Prof. Dr. Ong Hean Chooi	Jabatan Pergigian, Universiti Malaya	Fruit (LS1); leaf (LS2)
Lawsonia inermis (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (LI1)
Leptospermum flavescens (Smith, 1797)	Prof. Dr. Ong Hean Chooi	Genting Highlands, Pahang	Leaf (LF1); branch (LF2)
Macaranga conifera (Müller Argoviensis, 1866)	Prof. Dr. Ong Hean Chooi	Bukit Universiti Malaya	Branch (MC1)
Melaleuca cajuputi (Roxburgh, 1814)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Fruit & flower (MeC1)
Moringa oleifera (Lamarck and Poiret, 1785)	Prof. Dr. Ong Hean Chooi	Kebun ISB, Universiti Malaya	Branch (MO1)
Orthosiphon stamineus (Wallich, 1830)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Leaf (OS1)
Oxalis barrelieri (Linnaeus, 1762)	Prof. Dr. Ong Hean Chooi	Jabatan Kimia, Universiti Malaya	Root (OB1)
Peltophorum pterocarpum (Heyne, 1927)	Prof. Dr. Ong Hean Chooi	Pusat Sukan, Universiti Malaya	Fruit (PP1); leaf (PP2)
Pereskia bleo (Candolle, 1828)	Prof. Dr. Ong Hean Chooi	Kebun ISB, Universiti Malaya	Fruit (PB1)
Piper nigrum (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Rimba Ilmu, Universiti Malaya	Leaf (PN1); branch (PN2); fruit (PN3)
Scoparia dulcis (Linnaeus, 1753a)	Prof. Dr. Ong Hean Chooi	Sementa, Kuala Selangor	Root (SD1)

Table 7.1, continued

Plant sample	Authenticated by	Place of collection	Parts used (reference number)
Strobilanthes crispa (Blume, 1826b)	Prof. Dr. Ong Hean Chooi	Rimba Ilmu, Universiti Malaya	Branch (SC1)
Tetracera indica (Merrill, 1917)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Fruit (TI1)
Vitex sp. (Linnaeus, 1753b)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (V1); leaf (V2)
Vitex trifolia 'purpurea' (Linnaeus, 1753b)	Prof. Dr. Ong Hean Chooi	Pantai Dalam, Kuala Lumpur	Branch (VT1); leaf (VT2)

APPENDIX II: PREPARATION OF MEDIA, DILUENTS, BUFFERS AND SOLUTIONS

BASIC RPMI 1640 MEDIUM

To prepare 1 L of basic RPMI 1640 medium, 10.4 g of RPMI 1640 powder (with L-glutamine, without sodium bicarbonate), 2 g of sodium bicarbonate and 0.5206 g of HEPES were dissolved in 1 L of autoclaved distilled water and mixed well. pH of the medium was then adjusted to pH 7.4 with either sodium hydroxide or hydrochloric acid. The basic medium was then filtered and kept at 4 ℃ for further use.

10% SUPPLEMENTED RPMI 1640 MEDIUM

To prepare 100 mL of 10% supplemented RPMI 1640 medium, 90 mL of basic RPMI 1640 medium, 10 mL of heat-inactivated FBS, 1 mL of amphotericin B and 2 mL

of penicillin/streptomycin were mixed together. The mixture was then filtered and kept at 4 ℃.

20% SUPPLEMENTED RPMI 1640 MEDIUM

20% supplemented RPMI 1640 medium was prepared by added in 5 mL of heat-inactivated FBS into 50 mL of 10% supplemented RPMI 1640 medium. It was then filtered sterilized and kept at 4 ℃.

FREEZING MEDIUM

To prepare 10 mL of freezing medium, 4 mL of basic RPMI 1640 medium, 5 mL of heat-inactivated FBS and 1 mL of DMSO were mixed and filtered sterilized. The freezing medium was kept in 4 ℃ prior to use.

PHOSPHATE BUFFERED SALINE (PBS), pH 7.4

To prepare 1 L of 0.01M PBS (with 0.138M of NaCl; 0.0027M of KCl), one pouch of PBS powder was dissolved in 1 L of autoclaved distilled water and mixed well. pH of the solution was not adjusted. The solution was then filtered and autoclaved. Autoclaved PBS was kept at room temperature.

0.4% (w/v) TRYPAN BLUE

To prepare 50 mL of 0.4% trypan blue, 0.2 g of trypan blue is weighed and dissolved in 50 mL of distilled water.

25 mM SODIUM PHOSPHATE BUFFER, pH 7.4

To prepare 1 L of eluting buffer, 25 mM sodium phosphate buffer, 3 g of NaH_2PO_4 was dissolved in approximately 900 mL of distilled water. pH of the solution was adjusted to pH 7.4 at 20 ℃ and the volume was topped up to 1 L.

10 mM REDUCED GLUTATHIONE IN 25mM SODIUM PHOSPHATE BUFFER, pH 7.4

To prepare 100 mL of 10 mM reduced glutathione in 25 mM sodium phosphate buffer, pH 7.4, 0.307 g of reduced glutathione was dissolved in approximately 90 mL of 25 mM sodium phosphate buffer. pH of the solution was adjusted to pH 7.4 and the volume was then made up to 100 mL.

0.1 M SODIUM PHOSPHATE BUFFER, pH 6.5 (BUFFER A)

To prepare 1 L of buffer A, 12 g of NaH_2PO_4 was dissolved in approximately 900 mL of distilled water. The pH of the solution was adjusted to pH 6.5 and volume was then topped up to 1 L.

60 mM REDUCED GLUTATHIONE, pH 7.4

0.0184 g of reduced glutathione was weighed and dissolved in 1 mL of buffer A. The solution was made freshly and kept at 4 ℃.

60 mM 1-CHLORO-2,4-DINITROBENZENE (CDNB)

0.2430 g of 1-chloro-2,4-dinitrobenzene was weighed and dissolved in 20 mL of ethanol. The solution was kept in dark to avoid the exposure to light.

10% (w/v) SODIUM DODECYL SULFATE (SDS) SOLUTION

10% SDS solution was prepared by dissolved 10 g of SDS in 100 mL of de-ionized water.

10% (w/v) AMMONIUM PERSULFATE (APS) SOLUTION

10% APS solution was prepared freshly by dissolved 100 mg of APS in 1 mL of de-ionized water.

SDS RUNNING BUFFER, pH 8.3

To prepare 1 L of SDS running buffer, 3.03 g of Tris base, 14.40 g of glycine and 1.0 g of SDS were dissolved in 1 L of de-ionized water. The solution was kept at 4℃ prior to use.

SAMPLE BUFFER (SDS REDUCING BUFFER)

Sample buffer was prepared as shown in Table 7.2.

Table 7.2 : Sample buffer formulation

Solution	Volume (mL)
De-ionized water	3.55
0.5 M Tris-HCl, pH 6.8	1.25
Glycerol	2.50
10% (w/v) SDS	2.00
0.5% (w/v) Bromophenol blue	0.20

The sample buffer was stored at room temperature.

4% STACKING: 12% RESOLVING GEL MONOMER SOLUTIONS

Table 7.3 : Gel formulation

Solution	Stacking gel (4% acrylamide)	Resolving gel (12% acrylamide)
De-ionized water	6.1 mL	3.4 mL
30% Acrylamide/Bis	1.3 mL	4.0 mL
1.5 M Tris-HCl, pH 8.8	-	2.5 mL
0.5 M Tris-HCl, pH 6.8	2.5 mL	-
10% (w/v) SDS	0.1 mL	0.1 mL
10% (w/v) APS	50.0 µL	50.0 µL
TEMED	10.0 µL	5.0 µL

COLLOIDAL COOMASSIE BLUE SOLUTION

Table 7.4 : Colloidal coomassie blue formulation

Component	Amount
5% (w/v) brilliant blue G-250	20 mL
Ammonium sulfate, $(NH_4)_2SO_4$	100 g
Ortho-phosphoric acid, H_3PO_4	11.8 mL
Distilled water	~ 960 mL

5% brilliant blue G-250 solution was prepared by dissolved 1 g of brilliant blue G-250 in 20 mL of distilled water. To prepare 1 L of colloidal coomassie blue solution, 100 g of $(NH_4)_2SO_4$ was dissolved in 500 mL of distilled water. 11.8 mL of H_3PO_4 was then added into the $(NH_4)_2SO_4$ solution, followed by 5% brilliant blue solution added gradually into the mixture. The mixture was then topped up to 1 L with distilled water.

10% ACETONITRILE (ACN)

10% ACN was prepared by the dilution of 10 mL of 100% ACN with 90 mL of de-ionized water.

50% ACETONITRILE (ACN)

50% ACN was prepared by the dilution of 50 mL of 100% ACN with 50 mL of de-ionized water.

40 mM AMMONIUM BICARBONATE, $(NH_4)HCO_3$, IN 10% ACN

To prepare 5 mL of 40 mM $(NH_4)HCO_3$ in 10% ACN, 0.0158 g of $(NH_4)HCO_3$ was dissolved in 5 mL of 10% ACN.

200 mM AMMONIUM BICARBONATE, $(NH_4)HCO_3$, IN 50% ACN

To prepare 100 mL of 200 mM $(NH_4)HCO_3$ in 50% ACN, 1.58 g of $(NH_4)HCO_3$ was dissolved in 100 mL of 50% ACN.

0.1% TRIFLUOROACETIC ACID (TFA)

To prepare 10 mL of 0.1% TFA, 0.01 mL of 100% TFA was diluted with 9.99 mL of de-ionized water.

20 mg/mL OF α-CYANO-4-HYDROXYCINNAMIC ACID (CHCA) IN 0.1% TFA: 50% ACN SOLUTION (1: 1) (CHCA MATRIX SOLUTION)

To prepare 10 mL of matrix solution, 200 mg of CHCA was dissolved in 10 mL of 0.1%TFA: 50% ACN solution (5 mL of 0.1% TFA: 5 mL of 50% ACN).

2 mg/mL OF α-CYANO-4-HYDROXYCINNAMIC ACID (CHCA) IN ACN (DILUTED CHCA MATRIX SOLUTION)

Diluted CHCA matrix solution was prepared by dissolving 4 mg of CHCA in 2 mL of 100% ACN.

IEF ANODE BUFFER

To prepare 1 L of 1X anode buffer, 20 mL of 50X anode buffer is added to 980 mL of de-ionized water.

IEF CATHODE BUFFER

To prepare 200 mL of cathode buffer, 20 mL of 10X cathode buffer pH 3-10 is added to 180 mL of de-ionized water.

FIXING SOLUTION

To prepare 1 L of fixing solution, 120 mL of glacial acetic acid is added to 500 mL of methanol and 475 μL of formaldehyde. The final volume was brought up to 1 L with de-ionized water.

WASHING SOLUTION

To make 1 L of washing solution, 350 mL of ethanol is added to 650 mL of de-ionized water.

SENSITIZING SOLUTION

To prepare 1 L of sensitizing solution, 250 mg of sodium thiosulfate ($Na_2S_2O_3$) was added to small volume of de-ionized water, mixed well and brought up to the final volume of 1 L with de-ionized water.

STAINING SOLUTION

To prepare 1 L of staining solution, 2 g of silver nitrate ($AgNO_3$) and 720 µL of formaldehyde were added to a small volume of de-ionized water. Mixed well and brought up to 1 L with de-ionized water.

DEVELOPING SOLUTION

To make 1 L of developing solution, 60 g of sodium carbonate (Na_2CO_3) and 473 µL of formaldehyde were added to 20 mL of sensitizing solution. Mixed well and the final volume was brought up to 1 L with de-ionized water.

TERMINATING SOLUTION

To prepare 1 L of terminating solution, 120 mL of glacial acetic acid was added to 500 mL of methanol. The final volume was brought up to 1 L with de-ionized water.

PRESERVATION SOLUTION

To make 1 L of preservation solution, 10 mL of glacial acetic acid was added to 990 mL of de-ionized water.

12% TRICHLOROACETIC ACID

To prepare 100 mL of 12% trichloroacetic acid, 12 g of trichloroacetic acid was dissolved in 100 mL of de-ionized water.

2% ACETIC ACID

To prepare 1 L of 2% acetic acid, 20 mL of glacial acetic acid was added to 980 mL of de-ionized water.

50% METHANOL 2% ACETIC ACID

To prepare 1 L of 50% methanol 2% acetic acid, 500 mL of methanol was added to 20 mL of glacial acetic acid and brought up to 1 L with de-ionized water.

DILUENT X, DILUENT Y AND DILUENT Z

Basically, the diluents are tissue culture media with or without addition of DMSO. Diluent X is 10% supplemented Eagle Minimum Essential Medium (EMEM) prepared by EMEM containing L-glutamine, sodium bicarbonate, sodium pyruvate and HEPES, supplemented with 10% heat-inactivated FBS, 2% penicillin-streptomycin, 1% amphotericin B. Diluent Y was prepared by RPMI EMEM containing L-glutamine, sodium bicarbonate, sodium pyruvate and HEPES, supplemented with 10% heat-inactivated FBS, 2% penicillin-streptomycin, 1% amphotericin B and 0.2% DMSO while Diluent Z was prepared by EMEM containing L-glutamine, sodium bicarbonate, sodium pyruvate and HEPES, supplemented with 10% heat-inactivated FBS, 2% penicillin-streptomycin, 1% amphotericin B and 0.4% DMSO. All of the diluents were kept at 4 ℃.

MTT REAGENT

To prepared 1 mL of 5 mg/mL of MTT reagent, 5 mg of MTT was dissolved in 1 mL of PBS and filtered. The reagent was kept in dark at 4 ℃.

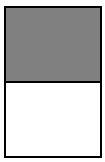
DILUENT A, DILUENT B AND DILUENT C

The diluents are tissue culture media with or without addition of DMSO. Diluent A is 10% supplemented RPMI 1640 medium prepared by RPMI 1640 medium containing L-glutamine, sodium bicarbonate and HEPES, supplemented with 10% heat-inactivated FBS, 2% penicillin-streptomycin, 1% amphotericin B. Diluent B was prepared by RPMI 1640 medium containing L-glutamine, sodium bicarbonate and HEPES, supplemented with 10% heat-inactivated FBS, 2% penicillin-streptomycin, 1% amphotericin B and 0.2% DMSO while Diluent C was prepared by RPMI 1640 medium containing L-glutamine, sodium bicarbonate and HEPES, supplemented with 10% heat-inactivated FBS, 2% penicillin-streptomycin, 1% amphotericin B and 0.4% DMSO. All of the diluents were kept at 4 ℃.

APPENDIX III: TEMPLATES FOR CYTOTOXICITY ASSAYS

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 7.1 : Template of serial dilution of sample used in cytotoxicity assays.



Sub-stock

Dilution

		Sample 1				Sample 2				Sample 3		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	100 µg/mL			Blank	100 µg/mL			Blank	100 µg/mL		
B		50 µg/mL				50 µg/mL				50 µg/mL		
C		25 µg/mL				25 µg/mL				25 µg/mL		
D		12.5 µg/mL				12.5 µg/mL				12.5 µg/mL		
E		6.25 µg/mL				6.25 µg/mL				6.25 µg/mL		
F		3.13 µg/mL				3.13 µg/mL				3.13 µg/mL		
G		1.56 µg/mL				1.56 µg/mL				1.56 µg/mL		
H		Negative control				Negative control				Negative control		

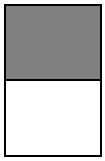
Figure 7.2 : Template of a 96-well microplate used for cytotoxicity assays with cells treated with plant samples (ethanolic extract/fraction) in a series of concentration (µg/mL), with final DMSO concentration of 0.2%.

		Ethacrynic acid				Cisplatin				Doxorubicin hydrochloride		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	100 µg/mL			Blank	10 µg/mL			Blank	10 µg/mL		
B		50 µg/mL				5 µg/mL				5 µg/mL		
C		25 µg/mL				2.5 µg/mL				2.5 µg/mL		
D		12.5 µg/mL				1.25 µg/mL				1.25 µg/mL		
E		6.25 µg/mL				0.625 µg/mL				0.625 µg/mL		
F		3.13 µg/mL				0.313 µg/mL				0.313 µg/mL		
G		1.56 µg/mL				0.156 µg/mL				0.156 µg/mL		
H		Negative control				Negative control				Negative control		

Figure 7.3 : Template of a 96-well microplate used for cytotoxicity assays with cells treated with drugs in a series of concentrations (µg/mL), with final DMSO concentration of 0.2%.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 7.4 : Template of serial dilution of drugs used in combination assay.



Sub-stock

Dilution

		Doxo + Plant sample				Doxo + EA				Doxo only		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	10 µg/mL + 50 µg/mL sample			Blank	10 µg/mL + 10 µg/mL EA			Blank	10 µg/mL		
B		5 µg/mL + 50 µg/mL sample				5 µg/mL + 10 µg/mL EA				5 µg/mL		
C		2.5 µg/mL + 50 µg/mL sample				2.5 µg/mL + 10 µg/mL EA				2.5 µg/mL		
D		1.25 µg/mL + 50 µg/mL sample				1.25 µg/mL + 10 µg/mL EA				1.25 µg/mL		
E		0.625 µg/mL + 50 µg/mL sample				0.625 µg/mL + 10 µg/mL EA				0.625 µg/mL		
F		0.313 µg/mL + 50 µg/mL sample				0.313 µg/mL + 10 µg/mL EA				0.313 µg/mL		
G		0.156 µg/mL + 50 µg/mL sample				0.156 µg/mL + 10 µg/mL EA				0.156 µg/mL		
H		Negative control				Negative control				Negative control		

Figure 7.5 : Template of a 96-well microplate used for combination assay with HT-29 cells treated with doxorubicin hydrochloride (Doxo) in a series of concentrations (µg/mL) with fixed concentration of plant sample (ethanolic extract/fraction) or ethacrynic acid (EA). Final concentration of DMSO was maintained at 0.2%.

		Cisplatin + Plant sample				Cisplatin + EA				Cisplatin only		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	10 µg/mL + 50 µg/mL sample			Blank	10 µg/mL + 10 µg/mL EA			Blank	10 µg/mL		
B		5 µg/mL + 50 µg/mL sample				5 µg/mL + 10 µg/mL EA				5 µg/mL		
C		2.5 µg/mL + 50 µg/mL sample				2.5 µg/mL + 10 µg/mL EA				2.5 µg/mL		
D		1.25 µg/mL + 50 µg/mL sample				1.25 µg/mL + 10 µg/mL EA				1.25 µg/mL		
E		0.625 µg/mL + 50 µg/mL sample				0.625 µg/mL + 10 µg/mL EA				0.625 µg/mL		
F		0.313 µg/mL + 50 µg/mL sample				0.313 µg/mL + 10 µg/mL EA				0.313 µg/mL		
G		0.156 µg/mL + 50 µg/mL sample				0.156 µg/mL + 10 µg/mL EA				0.156 µg/mL		
H		Negative control				Negative control				Negative control		

Figure 7.6 : Template of a 96-well microplate used for combination assay with HT-29 cells treated with cisplatin in a series of concentrations (µg/mL) with fixed concentration of plant sample (ethanolic extract/fraction) or ethacrynic acid (EA). Final concentration of DMSO was maintained at 0.2%.

APPENDIX IV: SOURCE OF DATA

GST-P IDENTIFICATION WITH MALDI-TOF

Mascot Search Results

Protein View

Match to: **GSTP1_HUMAN** Score: **184** Expect: **1e-013**
Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) (GSTP1-1) - Homo sapiens (Human)

Nominal mass (M_r): **23341**; Calculated pI value: **5.43**
NCBI BLAST search of GSTP1_HUMAN against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Homo sapiens

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **21%**

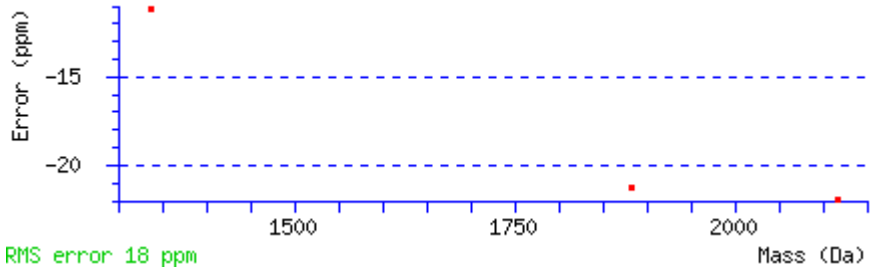
Matched peptides shown in **Bold Red**

```
     1 MPPYTVVYFP VRGRCAALRM LLADQGQSWK EEVVTVETWQ EGSLKASCLY
    51 GQLPKFQDGD LTLYQSNTIL RHLGRTLGLY GKDQQEAALV DMVNDGVEDL
   101 RCKYISLIYT NYEAGKDDYV KALPGQLKPF ETLLSQNQGG KTFIVGDQIS
   151 FADYNLLDLL LIHEVLAPGC LDAFPLLSAY VGRLSARPKL KAFLASPEYV
   201 NLPINGNGKQ
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
2 - 12	1337.71	1336.70	1336.72	-0.01	0	M.PPYTVVYFPVR.G (Ions score 59)
2 - 12	1337.71	1336.70	1336.72	-0.01	0	M.PPYTVVYFPVR.G (No match)
56 - 71	1883.91	1882.90	1882.94	-0.04	0	K.FQDGDLTLYQSNTILR.H (Ions score 72)
56 - 71	1883.91	1882.90	1882.94	-0.04	0	K.FQDGDLTLYQSNTILR.H (No match)
83 - 101	2116.94	2115.93	2115.97	-0.05	0	K.DQQEAALVDMVNDGVEDLR.C (Ions score 21)
83 - 101	2116.94	2115.93	2115.97	-0.05	0	K.DQQEAALVDMVNDGVEDLR.C (No match)

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ID   GSTP1_HUMAN             Reviewed;         210 AA.
AC   P09211; O00460; Q15690; Q5TZY3;
DT   01-JUL-1989, integrated into UniProtKB/Swiss-Prot.
DT   23-JAN-2007, sequence version 2.
DT   20-MAR-2007, entry version 94.
DE   Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) (GSTP1-
1).
GN   Name=GSTP1; Synonyms=GST3;
OS   Homo sapiens (Human).
OC   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC   Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
OC   Catarrhini; Hominidae; Homo.
OX   NCBI_TaxID=9606;
RN   [1]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RX   MEDLINE=88026724; PubMed=3664469;
RA   Kano T., Sakai M., Muramatsu M.;
RT   "Structure and expression of a human class pi glutathione S-
RT   transferase messenger RNA.";
RL   Cancer Res. 47:5626-5630(1987).
RN   [2]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA].
RX   MEDLINE=89061690; PubMed=3196325;
RA   Cowell I.G., Dixon K.H., Pemble S.E., Ketterer B., Taylor J.B.;
RT   "The structure of the human glutathione S-transferase pi gene.";
RL   Biochem. J. 255:79-83(1988).
RN   [3]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA].
RX   MEDLINE=89252918; PubMed=2542132; DOI=10.1016/0378-1119(89)90377-
6;
RA   Morrow C.S., Cowan K.H., Goldsmith M.E.;
RT   "Structure of the human genomic glutathione S-transferase-pi
gene.";
RL   Gene 75:3-11(1989).
RN   [4]
RP   NUCLEOTIDE SEQUENCE [MRNA].
RX   MEDLINE=89168166; PubMed=2466554;
RA   Moscow J.A., Fairchild C.R., Madden M.J., Ransom D.T., Wieand
H.S.,
RA   O'Brien E.E., Poplack D.G., Cossman J., Myers C.E., Cowan K.H.;
RT   "Expression of anionic glutathione-S-transferase and P-
glycoprotein
RT   genes in human tissues and tumors.";
RL   Cancer Res. 49:1422-1428(1989).
RN   [5]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA].
RA   Bora P.S., Smith C., Lange L.G., Bora N.S., Jones C., Gerhard
D.S.;
RT   "Human fatty acid ethyl ester synthase III gene: genomic
organization,
RT   nucleotide sequence, genetic and chromosomal sublocalization.";
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RL   Submitted (JUL-1994) to the EMBL/GenBank/DDBJ databases.
RN   [6]
RP   NUCLEOTIDE SEQUENCE [MRNA], AND VARIANTS VAL-105 AND VAL-114.
RX   MEDLINE=97248556; PubMed=9092542; DOI=10.1074/jbc.272.15.10004;
RA   Ali-Osman F., Akande O., Antoun G., Mao J.X., Buolamwini J.;
RT   "Molecular cloning, characterization, and expression in
Escherichia
RT   coli of full-length cDNAs of three human glutathione S-
transferase Pi
RT   gene variants. Evidence for differential catalytic activity of
the
RT   encoded proteins.";
RL   J. Biol. Chem. 272:10004-10012(1997).
RN   [7]
RP   NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA].
RA   Kalnine N., Chen X., Rolfs A., Halleck A., Hines L., Eisenstein
S.,
RA   Koundinya M., Raphael J., Moreira D., Kelley T., LaBaer J., Lin
Y.,
RA   Phelan M., Farmer A.;
RT   "Cloning of human full-length CDSs in BD Creator(TM) system donor
RT   vector.";
RL   Submitted (OCT-2004) to the EMBL/GenBank/DDBJ databases.
RN   [8]
RP   NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA].
RA   Ebert L., Schick M., Neubert P., Schatten R., Henze S., Korn B.;
RT   "Cloning of human full open reading frames in Gateway(TM) system
entry
RT   vector (pDONR201).";
RL   Submitted (MAY-2004) to the EMBL/GenBank/DDBJ databases.
RN   [9]
RP   NUCLEOTIDE SEQUENCE [GENOMIC DNA], AND VARIANTS VAL-105 AND VAL-
114.
RA   Rieder M.J., Livingston R.J., Daniels M.R., Chung M.-W.,
RA   Miyamoto K.E., Nguyen C.P., Nguyen D.A., Poel C.L., Robertson
P.D.,
RA   Schackwitz W.S., Sherwood J.K., Witrak L.A., Nickerson D.A.;
RT   "NIEHS-SNPs, environmental genome project, NIEHS ES15478,
Department
RT   of Genome Sciences, Seattle, WA (URL:
http://egp.gs.washington.edu).";
RL   Submitted (JUN-2003) to the EMBL/GenBank/DDBJ databases.
RN   [10]
RP   NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA], AND VARIANT VAL-105.
RC   TISSUE=Urinary bladder;
RX   PubMed=15489334; DOI=10.1101/gr.2596504;
RG   The MGC Project Team;
RT   "The status, quality, and expansion of the NIH full-length cDNA
RT   project: the Mammalian Gene Collection (MGC).";
RL   Genome Res. 14:2121-2127(2004).
RN   [11]
RP   PROTEIN SEQUENCE OF 2-24.
RX   MEDLINE=85154554; PubMed=3979555; DOI=10.1016/0014-5793(85)80324-
0;
RA   Alin P., Mannervik B., Joernvall H.;
RT   "Structural evidence for three different types of glutathione
RT   transferase in human tissues.";
RL   FEBS Lett. 182:319-322(1985).
RN   [12]
RP   PROTEIN SEQUENCE OF 2-24.
RX   MEDLINE=86042634; PubMed=3864155;
RA   Mannervik B., Alin P., Guthenberg C., Jensson H., Tahir M.K.,
RA   Warholm M., Joernvall H.;

RT "Identification of three classes of cytosolic glutathione transferase
RT common to several mammalian species: correlation between structural
RT data and enzymatic properties.";
RL Proc. Natl. Acad. Sci. U.S.A. 82:7202-7206(1985).
RN [13]
RP PROTEIN SEQUENCE OF 2-14.
RX MEDLINE=88280250; PubMed=3395118; DOI=10.1016/0003-9861(88)90564-4;
RA Singh S.V., Ahmad H., Kurosky A., Awasthi Y.C.;
RT "Purification and characterization of unique glutathione S-
RT transferases from human muscle.";
RL Arch. Biochem. Biophys. 264:13-22(1988).
RN [14]
RP PROTEIN SEQUENCE OF 2-12.
RC TISSUE=Platelet;
RX MEDLINE=22608298; PubMed=12665801; DOI=10.1038/nbt810;
RA Gevaert K., Goethals M., Martens L., Van Damme J., Staes A.,
RA Thomas G.R., Vandekerckhove J.;
RT "Exploring proteomes and analyzing protein processing by mass
RT spectrometric identification of sorted N-terminal peptides.";
RL Nat. Biotechnol. 21:566-569(2003).
RN [15]
RP PARTIAL PROTEIN SEQUENCE.
RC TISSUE=Colon carcinoma;
RX MEDLINE=97295306; PubMed=9150948;
RA Ji H., Reid G.E., Moritz R.L., Eddes J.S., Burgess A.W., Simpson R.J.;
RT "A two-dimensional gel database of human colon carcinoma proteins.";
RL Electrophoresis 18:605-613(1997).
RN [16]
RP PRIMARY AND SECONDARY STRUCTURAL ANALYZES.
RX MEDLINE=90225809; PubMed=2327795; DOI=10.1016/0003-9861(90)90277-6;
RA Ahmad H., Wilson D.E., Fritz R.R., Singh S.V., Medh R.D., Nagle G.T.,
RA Awasthi Y.C., Kurosky A.;
RT "Primary and secondary structural analyses of glutathione S-
RT transferase pi from human placenta.";
RL Arch. Biochem. Biophys. 278:398-408(1990).
RN [17]
RP X-RAY CRYSTALLOGRAPHY (2.8 ANGSTROMS).
RX MEDLINE=92395662; PubMed=1522586; DOI=10.1016/0022-2836(92)90692-D;
RA Reinemer P., Dirr H.W., Ladenstein R., Huber R., Lo Bello M.,
RA Federici G., Parker M.W.;
RT "Three-dimensional structure of class pi glutathione S-transferase
RT from human placenta in complex with S-hexylglutathione at 2.8-A
RT resolution.";
RL J. Mol. Biol. 227:214-226(1992).
RN [18]
RP X-RAY CRYSTALLOGRAPHY (1.9 ANGSTROMS).
RX MEDLINE=97164911; PubMed=9012673; DOI=10.1021/bi962316i;
RA Oakley A.J., Rossjohn J., Lo Bello M., Caccuri A.M., Federici G.,
RA Parker M.W.;
RT "The three-dimensional structure of the human Pi class glutathione
RT transferase P1-1 in complex with the inhibitor ethacrynic acid and its
RT glutathione conjugate.";
RL Biochemistry 36:576-585(1997).

```
RN   [19]
RP   X-RAY CRYSTALLOGRAPHY (1.8 ANGSTROMS).
RX   MEDLINE=97392635; PubMed=9245401; DOI=10.1021/bi970805s;
RA   Ji X., Tordova M., O'Donnell R., Parsons J.F., Hayden J.B.,
RA   Gilliland G.L., Zimniak P.;
RT   "Structure and function of the xenobiotic substrate-binding site
and
RT   location of a potential non-substrate-binding site in a class pi
RT   glutathione S-transferase.";
RL   Biochemistry 36:9690-9702(1997).
RN   [20]
RP   X-RAY CRYSTALLOGRAPHY (1.9 ANGSTROMS).
RX   MEDLINE=98062356; PubMed=9398518; DOI=10.1006/jmbi.1997.1364;
RA   Oakley A.J., Lo Bello M., Battistoni A., Ricci G., Rossjohn J.,
RA   Villar H.O., Parker M.W.;
RT   "The structures of human glutathione transferase P1-1 in complex
with
RT   glutathione and various inhibitors at high resolution.";
RL   J. Mol. Biol. 274:84-100(1997).
RN   [21]
RP   X-RAY CRYSTALLOGRAPHY (1.8 ANGSTROMS).
RX   MEDLINE=98035452; PubMed=9351803; DOI=10.1016/S0969-
2126(97)00281-5;
RA   Prade L., Huber R., Manoharan T.H., Fahl W.E., Reuter W.;
RT   "Structures of class pi glutathione S-transferase from human
placenta
RT   in complex with substrate, transition-state analogue and
inhibitor.";
RL   Structure 5:1287-1295(1997).
RN   [22]
RP   X-RAY CRYSTALLOGRAPHY (2.1 ANGSTROMS).
RX   MEDLINE=99371665; PubMed=10441116; DOI=10.1021/bi990668u;
RA   Ji X., Blaszczyk J., Xiao B., O'Donnell R., Hu X., Herzog C.,
RA   Singh S.V., Zimniak P.;
RT   "Structure and function of residue 104 and water molecules in the
RT   xenobiotic substrate-binding site in human glutathione S-
transferase
RT   P1-1.";
RL   Biochemistry 38:10231-10238(1999).
RN   [23]
RP   STRUCTURE BY NMR.
RX   MEDLINE=98153187; PubMed=9485454; DOI=10.1021/bi971902o;
RA   Nicotra M., Paci M., Sette M., Oakley A.J., Parker M.W., Lo Bello
M.,
RA   Caccuri A.M., Federici G., Ricci G.;
RT   "Solution structure of glutathione bound to human glutathione
RT   transferase P1-1: comparison of NMR measurements with the crystal
RT   structure.";
RL   Biochemistry 37:3020-3027(1998).
RN   [24]
RP   MUTAGENESIS.
RX   MEDLINE=93165656; PubMed=8433974; DOI=10.1093/protein/6.1.93;
RA   Kong K.-H., Inoue H., Takahashi K.;
RT   "Site-directed mutagenesis study on the roles of evolutionally
RT   conserved aspartic acid residues in human glutathione S-
transferase
RT   P1-1.";
RL   Protein Eng. 6:93-99(1993).
CC   -!- FUNCTION: Conjugation of reduced glutathione to a wide number
of
CC       exogenous and endogenous hydrophobic electrophiles.
CC   -!- CATALYTIC ACTIVITY: RX + glutathione = HX + R-S-glutathione.
CC   -!- SUBUNIT: Homodimer.
CC   -!- INTERACTION:
```

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CC       Q99683:MAP3K5; NbExp=1; IntAct=EBI-353467, EBI-476263;
CC       Q16539:MAPK14; NbExp=1; IntAct=EBI-353467, EBI-73946;
CC       Q12933:TRAF2; NbExp=3; IntAct=EBI-353467, EBI-355744;
CC   -!- SIMILARITY: Belongs to the GST superfamily. Pi family.
CC   -!- WEB RESOURCE: NAME=SHMPD;
CC       NOTE=The Singapore human mutation and polymorphism database;
CC       URL="http://shmpd.bii.a-
star.edu.sg/gene.php?genestart=A&genename=GSTP1".
CC   -----------------------------------------------------------------
------
CC   Copyrighted by the UniProt Consortium, see
http://www.uniprot.org/terms
CC   Distributed under the Creative Commons Attribution-NoDerivs
License
CC   -----------------------------------------------------------------
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DR   EMBL; X06547; CAA29794.1; -; mRNA.
DR   EMBL; M24485; AAA56823.1; -; Genomic_DNA.
DR   EMBL; X08058; CAA30847.1; -; Genomic_DNA.
DR   EMBL; X08094; CAA30894.1; -; Genomic_DNA.
DR   EMBL; X08095; CAA30894.1; JOINED; Genomic_DNA.
DR   EMBL; X08096; CAA30894.1; JOINED; Genomic_DNA.
DR   EMBL; X15480; CAA33508.1; -; mRNA.
DR   EMBL; U12472; AAA64919.1; -; Genomic_DNA.
DR   EMBL; U30897; AAC51280.1; -; mRNA.
DR   EMBL; U62589; AAC51237.1; -; mRNA.
DR   EMBL; U21689; AAC13869.1; -; Genomic_DNA.
DR   EMBL; BT019949; AAV38752.1; -; mRNA.
DR   EMBL; BT019950; AAV38753.1; -; mRNA.
DR   EMBL; CR450361; CAG29357.1; -; mRNA.
DR   EMBL; AY324387; AAP72967.1; -; Genomic_DNA.
DR   EMBL; BC010915; AAH10915.1; -; mRNA.
DR   PIR; JS0153; A37378.
DR   UniGene; Hs.523836; -.
DR   PDB; 10GS; X-ray; A/B=1-210.
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DR   PDB; 14GS; X-ray; A/B=1-210.
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DR   PDB; 1PX6; X-ray; A/B=1-210.
DR   PDB; 1PX7; X-ray; A/B=1-210.
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DR   PDB; 20GS; X-ray; A/B=1-210.
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DR   PDB; 2A2R; X-ray; A/B=1-210.
DR   PDB; 2A2S; X-ray; A/B=1-210.
DR   PDB; 2GSS; X-ray; A/B=1-210.
DR   PDB; 2J9H; X-ray; A/B=-.
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DR   PDB; 3GSS; X-ray; A/B=1-210.
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DR   PDB; 4GSS; X-ray; A/B=1-210.
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DR   PDB; 5GSS; X-ray; A/B=1-210.
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DR   PDB; 8GSS; X-ray; A/B/C=1-210.
DR   PDB; 9GSS; X-ray; A/B=1-210.
DR   IntAct; P09211; -.
DR   SWISS-2DPAGE; P09211; HUMAN.
DR   Aarhus/Ghent-2DPAGE; 5101; IEF.
DR   Cornea-2DPAGE; P09211; HUMAN.
DR   DOSAC-COBS-2DPAGE; P09211; HUMAN.
DR   HSC-2DPAGE; P09211; HUMAN.
DR   OGP; P09211; -.
DR   PHCI-2DPAGE; P09211; -.
DR   REPRODUCTION-2DPAGE; P09211; HUMAN.
DR   Siena-2DPAGE; P09211; -.
DR   Ensembl; ENSG00000084207; Homo sapiens.
DR   KEGG; hsa:2950; -.
DR   H-InvDB; HIX0009866; -.
DR   HGNC; HGNC:4638; GSTP1.
DR   MIM; 134660; gene.
DR   Reactome; REACT_2063.3; Xenobiotic metabolism.
DR   DrugBank; APRD00253; Clomipramine.
DR   LinkHub; P09211; -.
DR   ArrayExpress; P09211; -.
DR   GermOnline; ENSG00000084207; Homo sapiens.
DR   RZPD-ProtExp; IOH12222; -.
DR   RZPD-ProtExp; L0008; -.
DR   RZPD-ProtExp; RZPDo834C083; -.
DR   RZPD-ProtExp; RZPDo839D0264; -.
DR   RZPD-ProtExp; RZPDo839D0274; -.
DR   GO; GO:0005737; C:cytoplasm; TAS:UniProtKB.
DR   GO; GO:0005515; F:protein binding; IPI:IntAct.
DR   GO; GO:0006916; P:anti-apoptosis; TAS:UniProtKB.
DR   GO; GO:0007417; P:central nervous system development; TAS:ProtInc.
DR   InterPro; IPR004046; GST_C.
DR   InterPro; IPR010987; GST_C_like.
DR   InterPro; IPR004045; GST_N.
DR   InterPro; IPR003082; GST_pi.
DR   InterPro; IPR012336; Thiordxn-like_fd.
DR   InterPro; IPR012335; Thioredoxin_fold.
DR   Gene3D; G3DSA:3.40.30.10; Thioredoxin_fold; 1.
DR   Pfam; PF00043; GST_C; 1.
DR   Pfam; PF02798; GST_N; 1.
DR   PRINTS; PR01268; GSTRNSFRASEP.
KW   3D-structure; Direct protein sequencing; Polymorphism;
Transferase.
FT   INIT_MET      1      1       Removed.
FT   CHAIN         2    210       Glutathione S-transferase P.
FT                                /FTId=PRO_0000185900.
FT   VARIANT     105    105       I -> V (in allele GSTP1*B and allele
FT                                GSTP1*C; dbSNP:rs1695).
FT                                /FTId=VAR_014499.
FT   VARIANT     114    114       A -> V (in allele GSTP1*C;
FT                                dbSNP:rs1138272).
FT                                /FTId=VAR_014500.
FT   CONFLICT    186    186       A -> P (in Ref. 2).
FT   STRAND        3      7
FT   HELIX        12     14
FT   HELIX        15     23
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FT   STRAND       28     32
FT   HELIX        35     39
FT   HELIX        42     46
FT   STRAND       54     57
FT   STRAND       60     64
FT   HELIX        65     76
FT   HELIX        83    109
FT   HELIX       111    134
FT   HELIX       137    139
FT   STRAND      143    147
FT   HELIX       150    165
FT   TURN        167    172
FT   HELIX       174    184
FT   HELIX       187    194
FT   HELIX       196    199
SQ   SEQUENCE   210 AA;  23356 MW;  409E33FFAA338396 CRC64;
     MPPYTVVYFP VRGRCAALRM LLADQGQSWK EEVVTVETWQ EGSLKASCLY GQLPKFQDGD
     LTLYQSNTIL RHLGRTLGLY GKDQQEAALV DMVNDGVEDL RCKYISLIYT NYEAGKDDYV
     KALPGQLKPF ETLLSQNQGG KTFIVGDQIS FADYNLLDLL LIHEVLAPGC LDAFPLLSAY
     VGRLSARPKL KAFLASPEYV NLPINGNGKQ
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IC_{50} VALUE DETERMINATION WITH GST ACTIVITY ASSAYS

Figure 7.7 shows effects of *Anacardium occidentale* (branch) ethanolic extract on GST-P activity. The extract was inhibited the enzyme in dose-dependent manner. At the extract concentrations of 0.2 and 0.4 mg/mL, GST-P activities were inhibited 54 and 98% respectively. The IC_{50} value of *A. occidentale* (branch) ethanolic extract generated from the dose-response graph is 0.18 ± 0.006 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

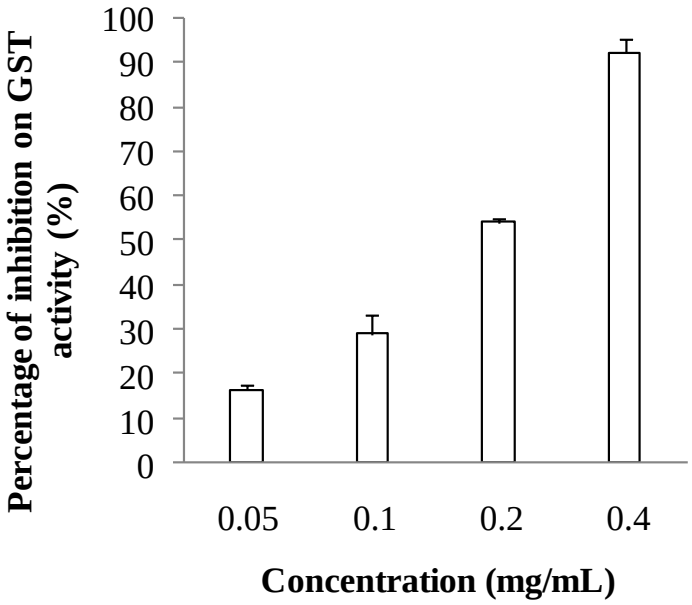


Figure 7.7 : Inhibitory effects of *A. occidentale* (branch) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Anacardium occidentale* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.154)

Equal Variance Test: Passed (P = 0.526)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	92.473	2.988	1.725
0.2	4	1	54.287	0.955	0.551
0.1	4	1	29.043	4.237	2.446
0.05	4	1	16.577	1.028	0.593
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	15643.943	3910.986	677.909	<0.001
Residual	10	57.692	5.769		
Total	14	15701.635			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	92.473	47.153	<0.001	Yes
0 vs. 0.2	54.287	27.681	<0.001	Yes
0 vs. 0.1	29.043	14.809	<0.001	Yes
0 vs. 0.05	16.577	8.453	<0.001	Yes

Figure 7.8 shows effects of *Andrographis paniculata* (leaf) ethanolic extract on GST-P activity. GST-P activity was inhibited dose-dependently by the extract up to 54%, at 0.4 mg/mL. The IC_{50} value of *A. paniculata* (leaf) ethanolic extract genenrated from the dose-response graph is 0.32 ± 0.021 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

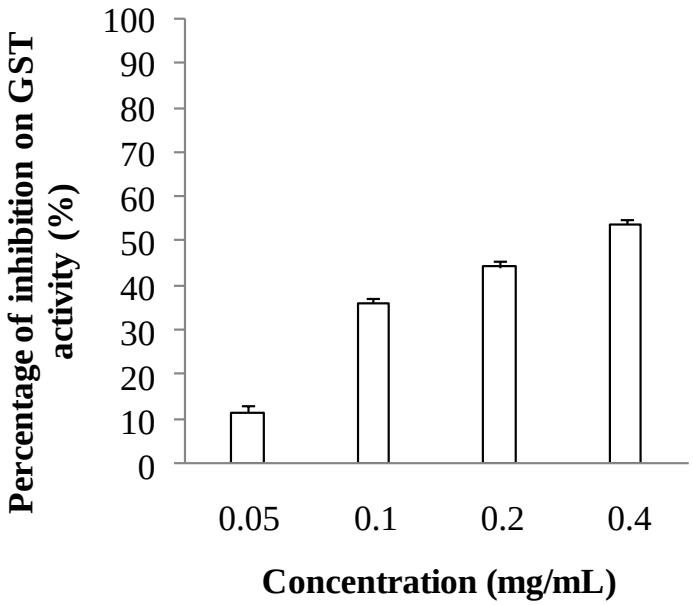


Figure 7.8 : Inhibitory effects of *A. paniculata* (leaf) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Andrographis paniculata* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.395)

Equal Variance Test: Passed (P = 0.153)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	54.067	0.955	0.551
0.2	4	1	44.443	1.097	0.633
0.1	4	1	36.170	1.299	0.750
0.05	4	1	11.670	1.529	0.883
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	6177.623	1544.406	1257.748	<0.001
Residual	10	12.279	1.228		
Total	14	6189.902			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	54.067	59.757	<0.001	Yes
0 vs. 0.2	44.443	49.121	<0.001	Yes
0 vs. 0.1	36.170	39.977	<0.001	Yes
0 vs. 0.05	11.670	12.898	<0.001	Yes

Figure 7.9 shows effects of *Cinnamomum zeylanicum* (branch) ethanolic extract on GST-P activity. GST-P activity was inhibited in dose-dependent manner by the extract. Inhibition at the concentrations of 0.1, 0.2 and 0.4 mg/mL are higher than 50%, which is 66, 75 and 80% respectively. The IC_{50} value of *C. zeylanicum* (branch) ethanolic extract generated from the dose-response graph is 0.07 ± 0.006 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

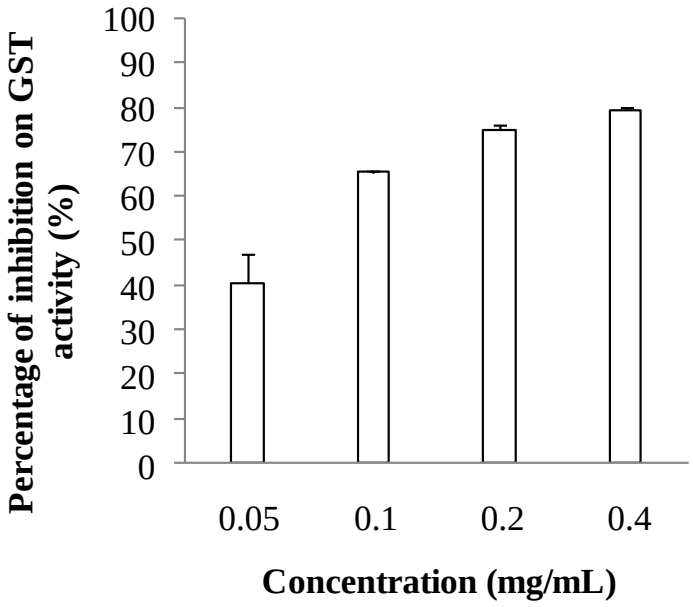


Figure 7.9 : Inhibitory effects of *C. zeylanicum* (branch) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Cinnamomum zeylanicum* (branch)

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	79.540	0.930	0.537
0.2	4	1	75.223	0.885	0.511
0.1	4	1	65.543	0.647	0.373
0.05	4	1	40.740	6.577	3.797
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	12935.296	3233.824	356.793	<0.001
Residual	10	90.636	9.064		
Total	14	13025.931			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	79.540	32.358	<0.001	Yes
0 vs. 0.2	75.223	30.602	<0.001	Yes
0 vs. 0.1	65.543	26.664	<0.001	Yes
0 vs. 0.05	40.740	16.574	<0.001	Yes

Figure 7.10 shows effects of *Euodia redlevi* (fruit&flower) ethanolic extract on GST-P activity. GST-P activity was inhibited in dose-dependent manner by the extract. Inhibition at the concentrations of 0.2, 0.4 and 0.8 mg/mL are higher than 50%, which is 52, 63 and 65% respectively. However, at concentration higher than 0.4 mg/mL, the inhibition seems to be not much different. The IC_{50} value of *E. redleri* (fruit&flower) ethanolic extract generated from the dose-response graph is 0.19 ± 0.031 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

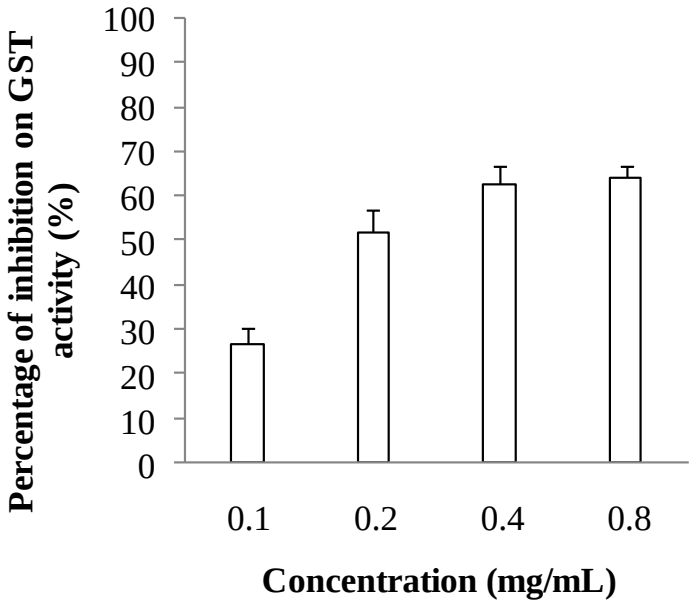


Figure 7.10 : Inhibitory effects of *E. redleri* (fruit&flower) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Euodia redlevi* (fruit&flower) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.639)

Equal Variance Test: Passed (P = 0.822)

Group Name	N	Missing	Mean	Std Dev	SEM
0.8	4	1	64.467	2.535	1.463
0.4	4	1	62.780	3.945	2.277
0.2	4	1	52.100	4.790	2.766
0.1	4	1	26.827	3.518	2.031
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	9089.613	2272.403	198.271	<0.001
Residual	10	114.611	11.461		
Total	14	9204.223			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.8	64.467	23.322	<0.001	Yes
0 vs. 0.4	62.780	22.712	<0.001	Yes
0 vs. 0.2	52.100	18.848	<0.001	Yes
0 vs. 0.1	26.827	9.705	<0.001	Yes

Figure 7.11 shows effects of *Garcinia atroviridis* (branch) ethanolic extract on GST-P activity. GST-P activity was dose-dependently inhibited by the extract up to 93%, at 0.4 mg/mL. The inhibition at the concentrations of 0.05 and 0.1 mg/mL are lower than 50%, which is 21 and 39% respectively. However, at concentration of 0.2 mg/mL, the inhibition observed was 60%. The IC_{50} value of *G. atroviridis* (branch) ethanolic extract generated from the dose-response graph is 0.16 ± 0.005 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

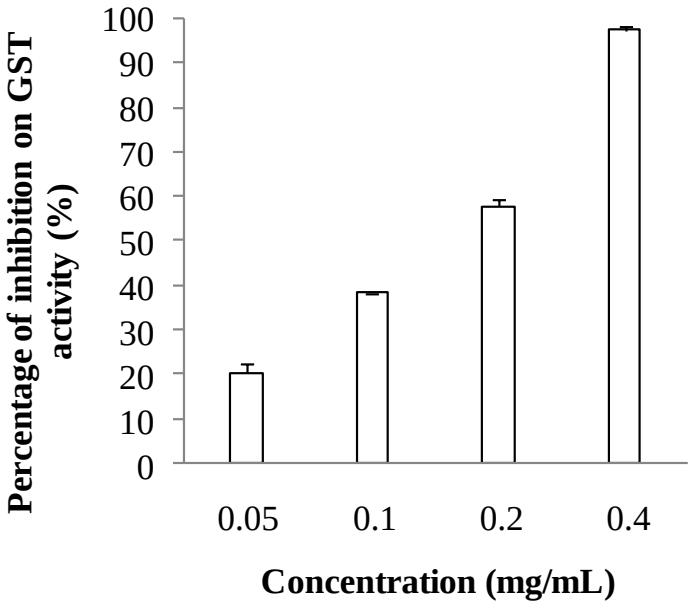


Figure 7.11 : Inhibitory effects of *G. atroviridis* (branch) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia atroviridis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.094)

Equal Variance Test: Passed (P = 0.627)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	97.580	0.942	0.544
0.2	4	1	57.987	1.593	0.920
0.1	4	1	38.610	0.000	0.000
0.05	4	1	20.513	2.002	1.156
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	16732.439	4183.110	2813.436	<0.001
Residual	10	14.868	1.487		
Total	14	16747.307			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	97.580	98.011	<0.001	Yes
0 vs. 0.2	57.987	58.243	<0.001	Yes
0 vs. 0.1	38.610	38.781	<0.001	Yes
0 vs. 0.05	20.513	20.604	<0.001	Yes

Figure 7.12 shows effects of *Garcinia mangostana* (branch) ethanolic extract on GST-P activity. With the addition of this extract, GST-P activity was inhibited in dose-dependent manner. The inhibition at the concentrations of 0.2 and 0.4 mg/mL are higher than 50%, which is 72 and 93% respectively. However, at concentration of 0.05 and 0.1 mg/mL, the inhibition observed was only 36 and 43% respectively. The IC_{50} value of *G. mangostana* (branch) ethanolic extract generated from the dose-response graph is 0.12 ± 0.002 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

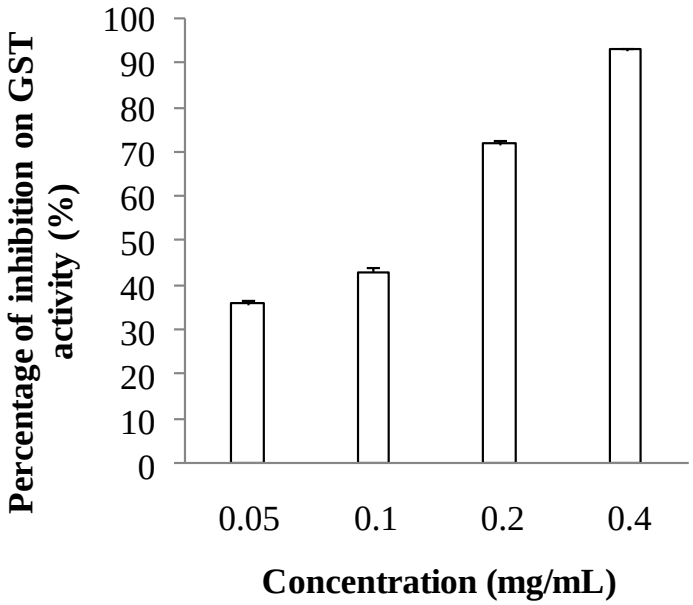


Figure 7.12 : Inhibitory effects of *G. mangostana* (branch) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.214)

Equal Variance Test: Passed (P = 0.057)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	93.120	0.422	0.244
0.2	4	1	72.090	0.930	0.537
0.1	4	1	43.120	0.920	0.531
0.05	4	1	36.100	0.636	0.367
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	15244.926	3811.231	8306.956	<0.001
Residual	10	4.588	0.459		
Total	14	15249.514			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	93.120	168.375	<0.001	Yes
0 vs. 0.2	72.090	130.349	<0.001	Yes
0 vs. 0.1	43.120	77.967	<0.001	Yes
0 vs. 0.05	36.100	65.274	<0.001	Yes

Figure 7.13 shows effects of *Garcinia mangostana* (leaf) ethanolic extract on GST-P activity. With the addition of this extract, GST-P activity was inhibited in dose-dependent manner. The inhibition at the concentrations of 0.2 and 0.4 mg/mL are higher than 50%, which is 67 and 77% respectively. However, at concentration of 0.05 and 0.1 mg/mL, the inhibition observed was only 32 and 50% respectively. The IC_{50} value of *G. mangostana* (leaf) ethanolic extract generated from the dose-response graph is 0.11 ± 0.006 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

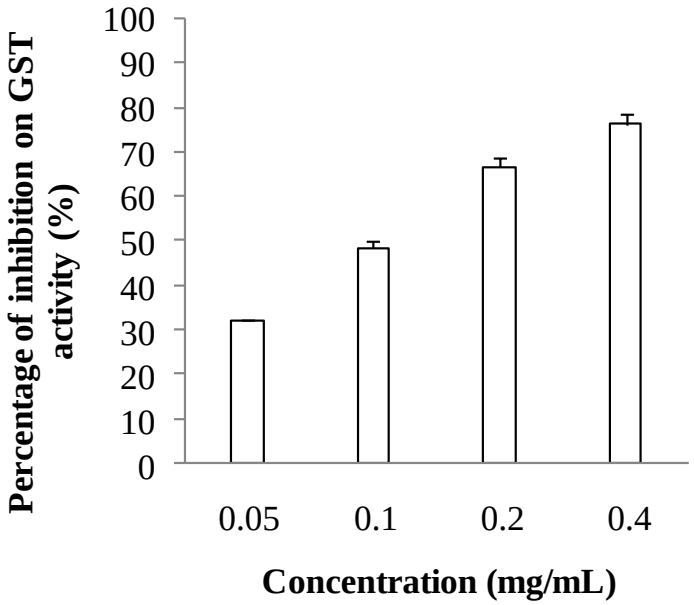


Figure 7.13 : Inhibitory effects of *G. mangostana* (leaf) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.377)

Equal Variance Test: Passed (P = 0.423)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	76.480	2.105	1.215
0.2	4	1	66.803	2.054	1.186
0.1	4	1	48.597	1.391	0.803
0.05	4	1	32.370	0.312	0.180
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	10991.148	2747.787	1286.108	<0.001
Residual	10	21.365	2.137		
Total	14	11012.514			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	76.480	64.083	<0.001	Yes
0 vs. 0.2	66.803	55.975	<0.001	Yes
0 vs. 0.1	48.597	40.719	<0.001	Yes
0 vs. 0.05	32.370	27.123	<0.001	Yes

Figure 7.14 shows effects of *Hibiscus tiliaceus* (leaf) ethanolic extract on GST-P activity. The extract inhibited GST-P activity in dose-dependent manner. Inhibition at the concentrations of 0.1, 0.2 and 0.4 mg/mL are higher than 50%, which is 50, 71 and 79% respectively. However, the inhibition at 0.05 mg/mL was only 31%. The IC_{50} value of *H. tiliaceus* (leaf) ethanolic extract generated from the dose-response graph is 0.10 ± 0.000 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

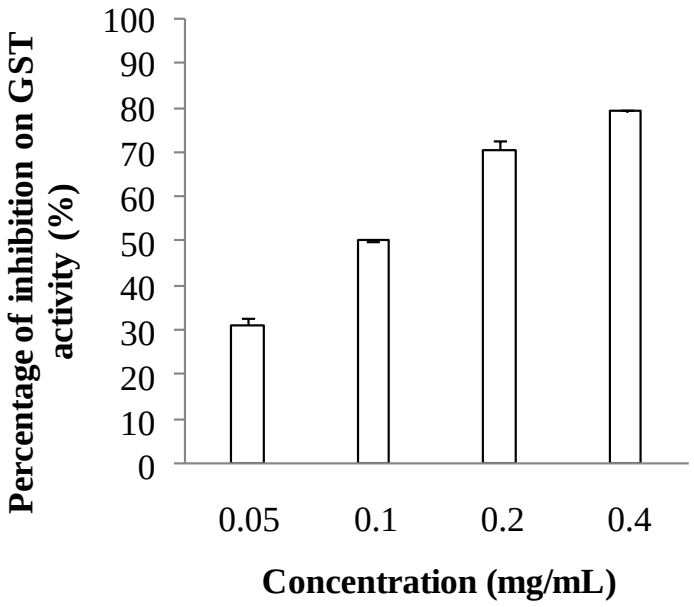


Figure 7.14 : Inhibitory effects of *H. tiliaceus* (leaf) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Hibiscus tiliaceus* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.060)

Equal Variance Test: Passed (P = 0.395)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	79.273	0.682	0.394
0.2	4	1	70.870	1.873	1.082
0.1	4	1	50.240	0.000	0.000
0.05	4	1	31.330	1.720	0.993
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	12222.693	3055.673	2203.388	<0.001
Residual	10	13.868	1.387		
Total	14	12236.561			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	79.273	82.445	<0.001	Yes
0 vs. 0.2	70.870	73.706	<0.001	Yes
0 vs. 0.1	50.240	52.250	<0.001	Yes
0 vs. 0.05	31.330	32.584	<0.001	Yes

Figure 7.15 shows effects of *Lawsonia inermis* (branch) ethanolic extract on GST-P activity. The extract inhibited GST-P activity in dose-dependent manner up to 85%, at 1.6 mg/mL. Inhibition at the concentration of 0.8 mg/mL is 65%, while the inhibition at 0.2 and 0.4 mg/mL were lower than 50%, which are 23 and 46% respectively. The IC_{50} value of *L. inermis* (branch) ethanolic extract generated from the dose-response graph is 0.46 ± 0.042 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

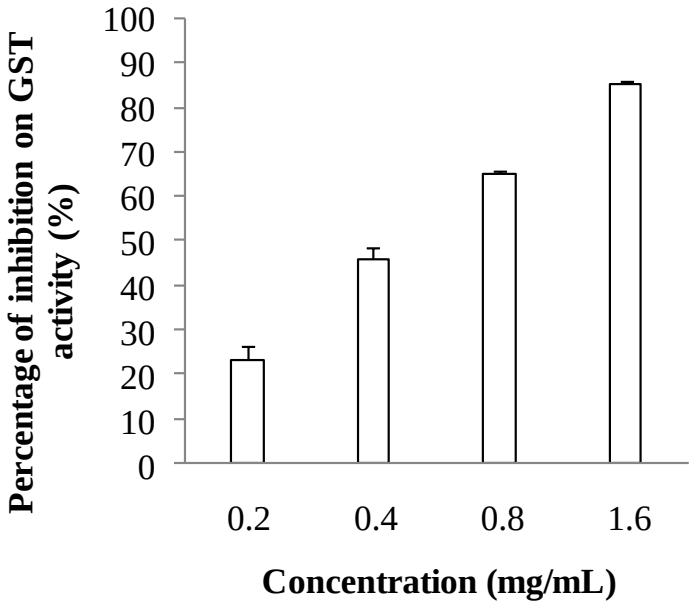


Figure 7.15 : Inhibitory effects of *L. inermis* (branch) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Lawsonia inermis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.088)

Equal Variance Test: Passed (P = 0.510)

Group Name	N	Missing	Mean	Std Dev	SEM
1.6	4	1	85.420	0.901	0.520
0.8	4	1	65.320	0.779	0.450
0.4	4	1	46.010	2.935	1.695
0.2	4	1	23.477	2.930	1.691
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	13594.188	3398.547	912.801	<0.001
Residual	10	37.232	3.723		
Total	14	13631.420			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1.6	85.420	54.218	<0.001	Yes
0 vs. 0.8	65.320	41.460	<0.001	Yes
0 vs. 0.4	46.010	29.204	<0.001	Yes
0 vs. 0.2	23.477	14.901	<0.001	Yes

Figure 7.16 shows effects of *Leptospermum flavescens* (leaf) ethanolic extract on GST-P activity. The extract inhibited GST-P activity in dose-dependent manner. The inhibition at the concentrations of 0.1 and 0.2 mg/mL are higher than 50%, which is 53 and 69% respectively. However, at concentration of 0.025 and 0.05 mg/mL, the inhibition observed was only 28 and 40% respectively. The IC_{50} value of *L. flavescens* (leaf) ethanolic extract generated from the dose-response graph is 0.09 ± 0.002 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

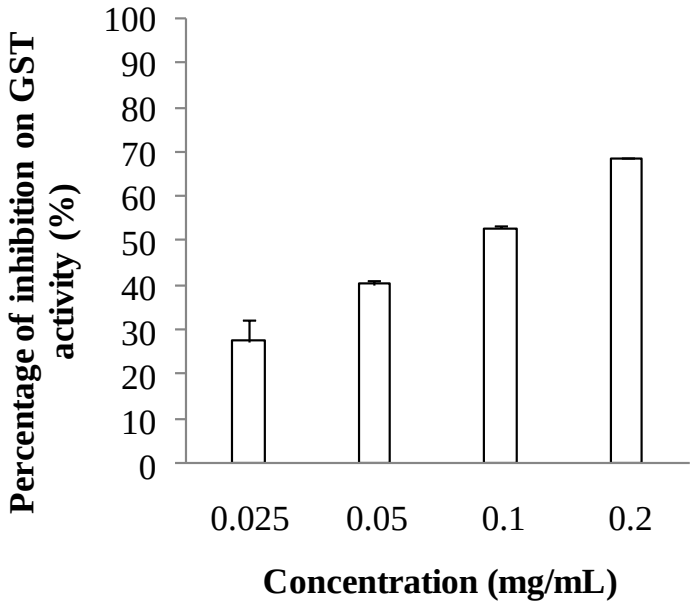


Figure 7.16 : Inhibitory effects of *L. flavescens* (leaf) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Leptospermum flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	68.780	0.000	0.000
0.1	4	1	53.117	0.525	0.303
0.05	4	1	40.397	0.981	0.567
0.025	4	1	27.620	4.641	2.680
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	8200.247	2050.062	449.913	<0.001
Residual	10	45.566	4.557		
Total	14	8245.813			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	68.780	39.463	<0.001	Yes
0 vs. 0.1	53.117	30.476	<0.001	Yes
0 vs. 0.05	40.397	23.178	<0.001	Yes
0 vs. 0.025	27.620	15.847	<0.001	Yes

Figure 7.17 shows effects of *Oxalis barrelieri* (root) ethanolic extract on GST-P activity. The extract inhibited GST-P activity in dose-dependent manner. The inhibition at the concentrations of 0.2 and 0.4 mg/mL are higher than 50%, which is 62 and 76% respectively. However, at concentration of 0.05 and 0.1 mg/mL, the inhibition observed was only 33 and 38% respectively. The IC_{50} value of *O. barrelieri* (root) ethanolic extract generated from the dose-response graph is 0.15 ± 0.012 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

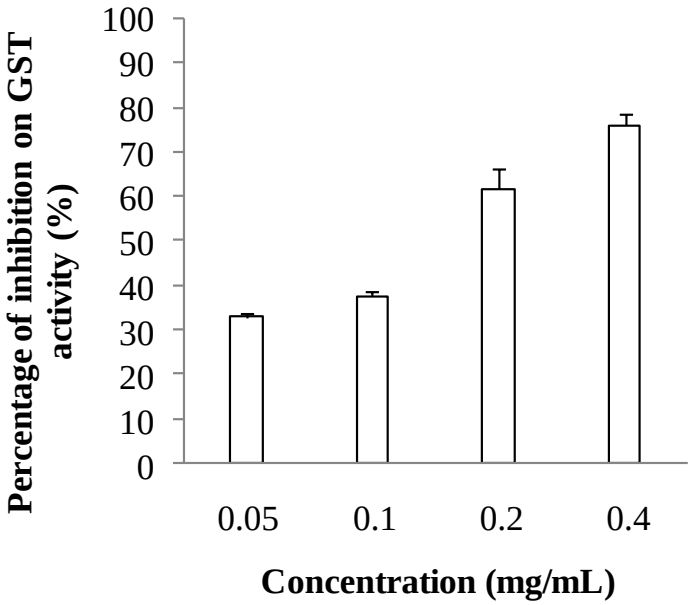


Figure 7.17 : Inhibitory effects of *O. barrelieri* (root) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Oxalis barrelieri* (root) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.547)

Equal Variance Test: Passed (P = 0.228)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	76.233	2.330	1.345
0.2	4	1	61.907	4.316	2.492
0.1	4	1	37.653	1.415	0.817
0.05	4	1	33.000	1.179	0.681
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	10295.396	2573.849	468.837	<0.001
Residual	10	54.899	5.490		
Total	14	10350.295			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	76.233	39.848	<0.001	Yes
0 vs. 0.2	61.907	32.360	<0.001	Yes
0 vs. 0.1	37.653	19.682	<0.001	Yes
0 vs. 0.05	33.000	17.250	<0.001	Yes

Figure 7.18 shows effects of *Peltophorum pterocarpum* (leaf) ethanolic extract on GST-P activity. The extract inhibited GST-P activity in dose-dependent manner. The inhibition at the concentrations of 0.4 and 0.8 mg/mL are higher than 50%, which is 58 and 98% respectively. However, at concentration of 0.1 and 0.2 mg/mL, the inhibition observed was only 11 and 45% respectively. The IC_{50} value of *P. pterocarpum* (leaf) ethanolic extract generated from the dose-response graph is 0.25 ± 0.025 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

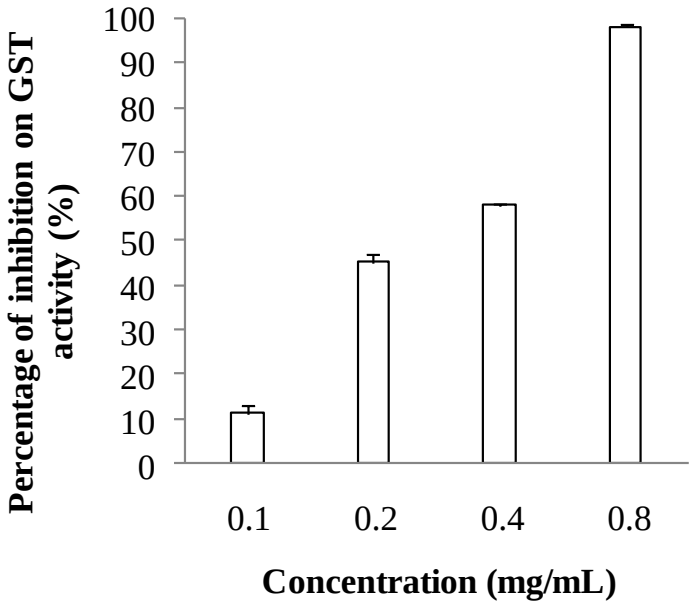


Figure 7.18 : Inhibitory effects of *P. pterocarpum* (leaf) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Peltophorum pterocarpum* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.260)

Equal Variance Test: Passed (P = 0.322)

Group Name	N	Missing	Mean	Std Dev	SEM
0.8	4	1	98.250	0.650	0.375
0.4	4	1	58.177	0.543	0.313
0.2	4	1	45.317	1.921	1.109
0.1	4	1	11.303	1.735	1.002
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	18423.529	4605.882	3105.159	<0.001
Residual	10	14.833	1.483		
Total	14	18438.362			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.8	98.250	98.802	<0.001	Yes
0 vs. 0.4	58.177	58.503	<0.001	Yes
0 vs. 0.2	45.317	45.571	<0.001	Yes
0 vs. 0.1	11.303	11.367	<0.001	Yes

Figure 7.19 shows effects of *Tetracera indica* (fruit) ethanolic extract on GST-P activity. The extract inhibited GST-P activity in dose-dependent manner. The inhibition at the concentrations of 0.2 and 0.4 mg/mL are higher than 50%, which is 63 and 79% respectively. However, at concentration of 0.05 and 0.1 mg/mL, the inhibition observed was only 34 and 46% respectively. The IC_{50} value of *T. indica* (fruit) ethanolic extract generated from the dose-response graph is 0.12 ± 0.006 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

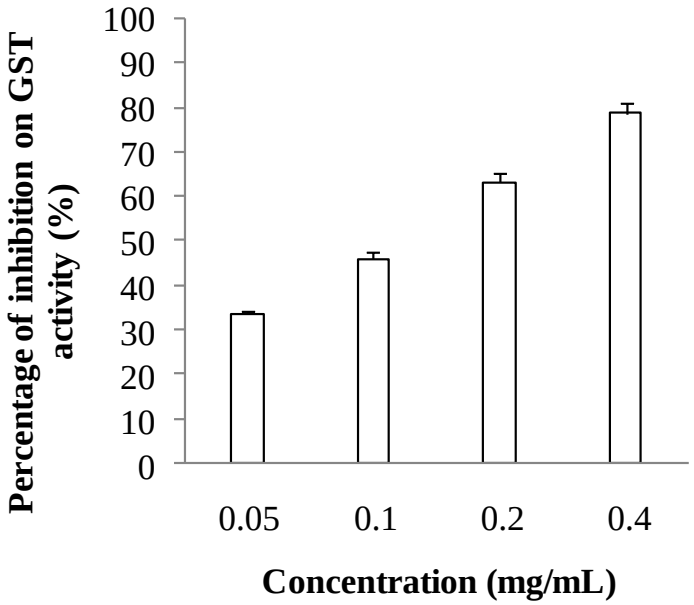


Figure 7.19 : Inhibitory effects of *T. indica* (fruit) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Tetracera indica* (fruit) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.202)

Equal Variance Test: Passed (P = 0.512)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	78.983	2.050	1.183
0.2	4	1	63.363	2.270	1.311
0.1	4	1	46.140	1.502	0.867
0.05	4	1	33.890	0.485	0.280
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	10921.303	2730.326	1152.273	<0.001
Residual	10	23.695	2.370		
Total	14	10944.998			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	78.983	62.842	<0.001	Yes
0 vs. 0.2	63.363	50.414	<0.001	Yes
0 vs. 0.1	46.140	36.711	<0.001	Yes
0 vs. 0.05	33.890	26.964	<0.001	Yes

Figure 7.20 shows effects of 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on GST-P activity. The fraction inhibited GST-P activity in dose-dependent manner. The inhibition at the concentrations of 0.2 and 0.4 mg/mL are higher than 50%, which is 53 and 92% respectively. However, at concentration of 0.05 and 0.1 mg/mL, the inhibition observed was only 6 and 18% respectively. The IC_{50} value of 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract generated from the dose-response graph is 0.19 ± 0.004 mg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

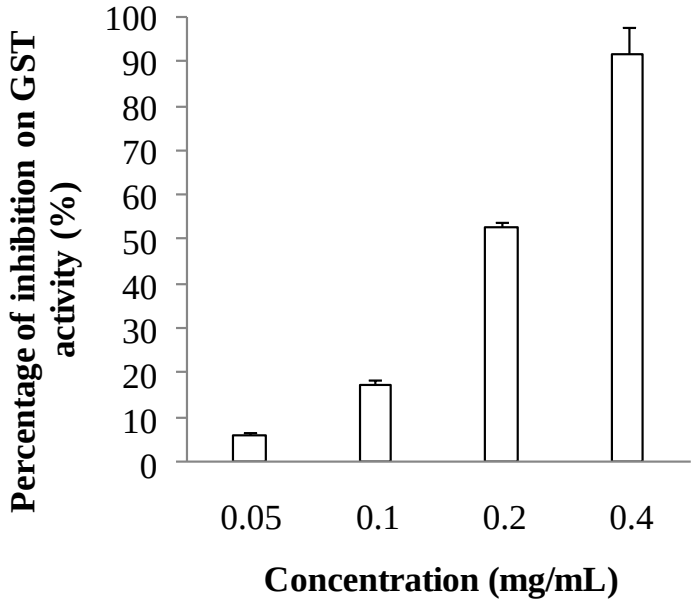


Figure 7.20 : Inhibitory effects of 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on GST-P. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
0.4	4	1	91.933	6.004	3.467
0.2	4	1	53.127	1.045	0.603
0.1	4	1	17.447	1.201	0.693
0.05	4	1	6.220	0.697	0.402
0	4	1	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	4	17770.552	4442.638	568.509	<0.001
Residual	10	78.145	7.815		
Total	14	17848.698			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 0.4	91.933	40.278	<0.001	Yes
0 vs. 0.2	53.127	23.276	<0.001	Yes
0 vs. 0.1	17.447	7.644	<0.001	Yes
0 vs. 0.05	6.220	2.725	0.021	Yes

One Way Analysis of Variance

Data source: Overall IC50

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.131)

Group Name	N	Missing	Mean	Std Dev	SEM
Anacardium occidentale (branch	3	0	0.182	0.00624	0.00361
Andrographis paniculata (leaf)	3	0	0.315	0.0210	0.0121
Cinnamomum zeylanicum (bran	3	0	0.0650	0.00624	0.00361
Euodia redlevi (fruit&flower)	3	0	0.194	0.0309	0.0179
Garcinia atroviridis (branch)	3	0	0.159	0.00458	0.00265
Garcinia mangostana (branch)	3	0	0.124	0.00200	0.00115
Garcinia mangostana (leaf)	3	0	0.107	0.00608	0.00351
Hibiscus tiliaceus (leaf)	3	0	0.0990	0.000	0.000
Lawsonia inermis (branch)	3	0	0.460	0.0418	0.0241
Leptospermum flavescens (leaf)	3	0	0.0843	0.00153	0.000882
Oxalis barrelieri (root)	3	0	0.146	0.0122	0.00702
Peltophorum pterocarpum (leaf)	3	0	0.253	0.0252	0.0146
Tetracera indica (fruit)	3	0	0.122	0.00603	0.00348
50% MeOH 2% acetic acid fracti	3	0	0.189	0.00416	0.00240
0	3	0	0.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	14	0.524	0.0374	136.123	<0.001
Residual	30	0.00824	0.000275		
Total	44	0.532			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

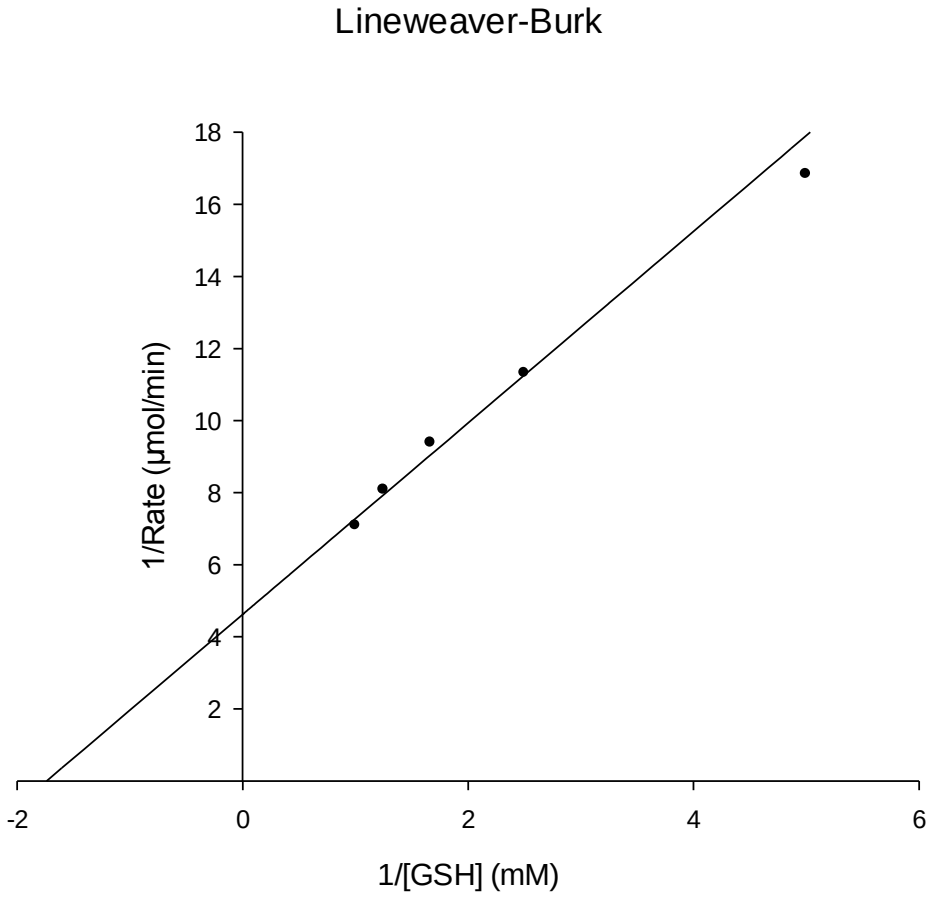
Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. Lawsonia ine	0.460	34.016	<0.001	Yes
0 vs. Andrographis	0.315	23.277	<0.001	Yes
0 vs. Peltophorum	0.253	18.695	<0.001	Yes
0 vs. Euodia redle	0.194	14.311	<0.001	Yes
0 vs. 50% MeOH 2%	0.189	13.941	<0.001	Yes
0 vs. Anacardium o	0.182	13.449	<0.001	Yes
0 vs. Garcinia atr	0.159	11.749	<0.001	Yes
0 vs. Oxalis barrelieri (root)	0.146	10.788	<0.001	Yes
0 vs. Garcinia man	0.124	9.163	<0.001	Yes
0 vs. Tetracera indica (fruit)	0.122	8.990	<0.001	Yes

0 vs. Garcinia man	0.107	7.907	<0.001	Yes
0 vs. Hibiscus til	0.0990	7.315	<0.001	Yes
0 vs. Leptospermum	0.0843	6.232	<0.001	Yes
0 vs. Cinnamomum z	0.0650	4.803	<0.001	Yes

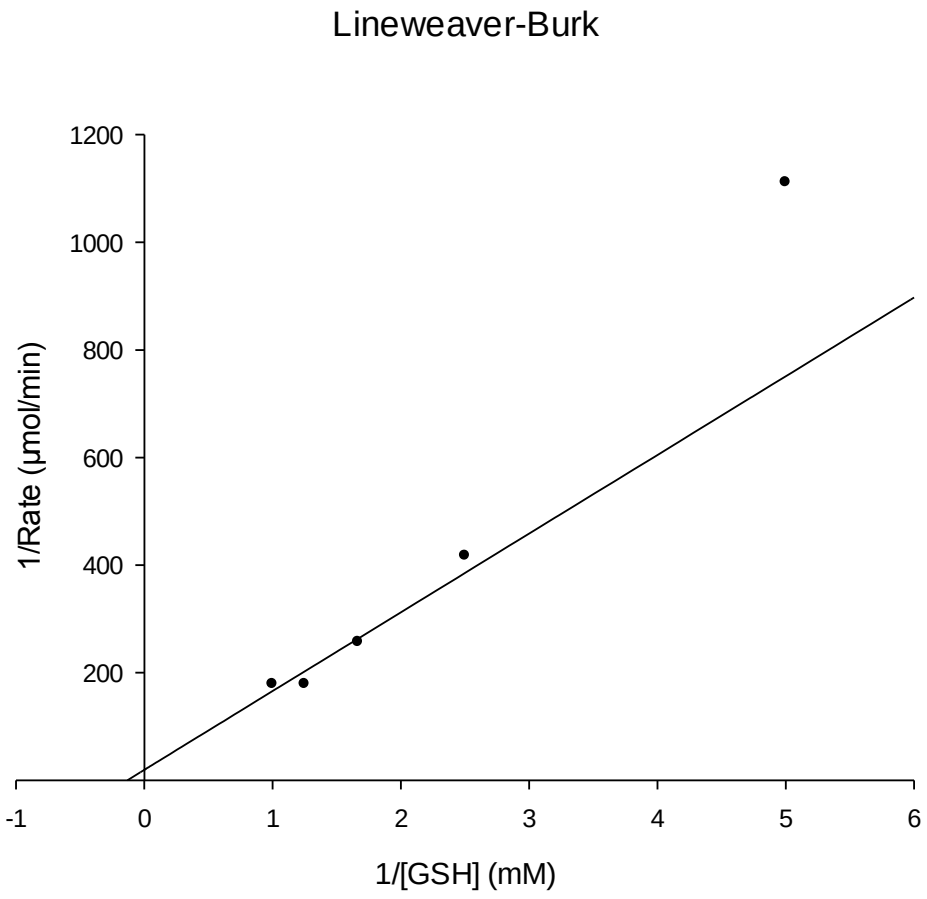
KINETIC STUDIES

Kinetics of GST-P alone



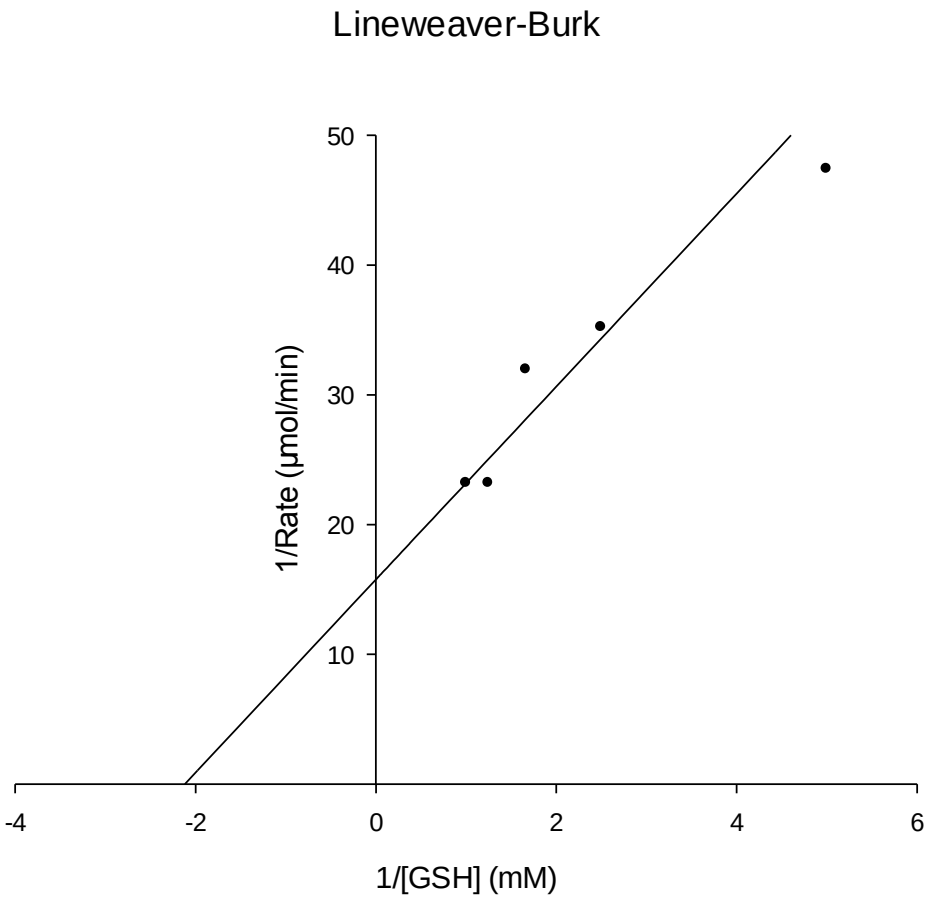
Vmax = 0.2163
Km = 0.5746

Kinetics of GST-P in the presence of *Anacardium occidentale* (branch) ethanolic extract



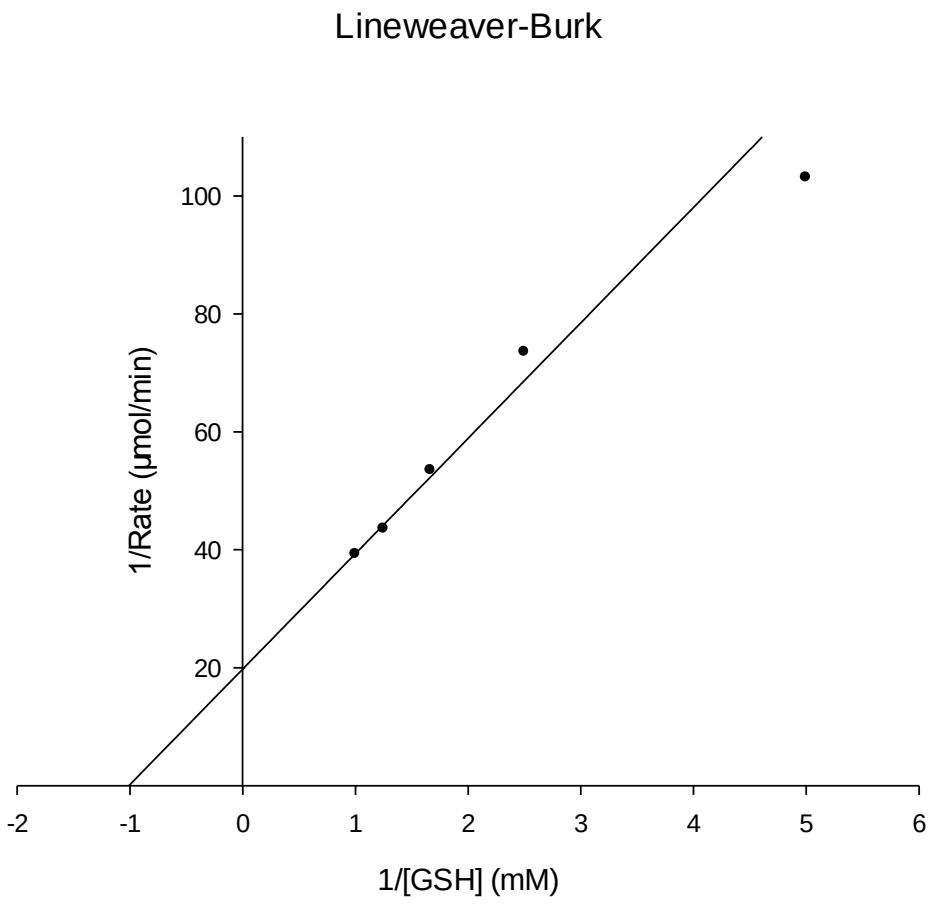
Vmax = 0.05105
Km = 7.5

Kinetics of GST-P in the presence of *Andrographis paniculata* (leaf) ethanolic extract



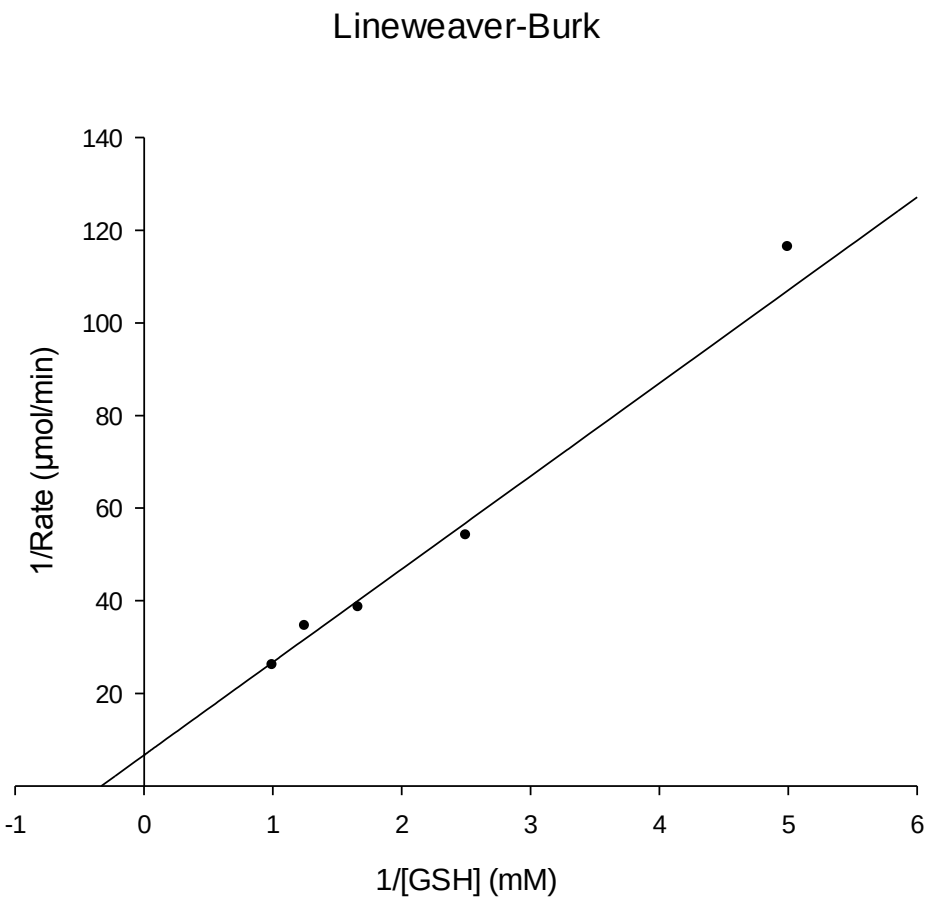
Vmax = 0.06341
Km = 0.4715

Kinetics of GST-P in the presence of *Cinnamomum zeylanicum* (branch) ethanolic extract



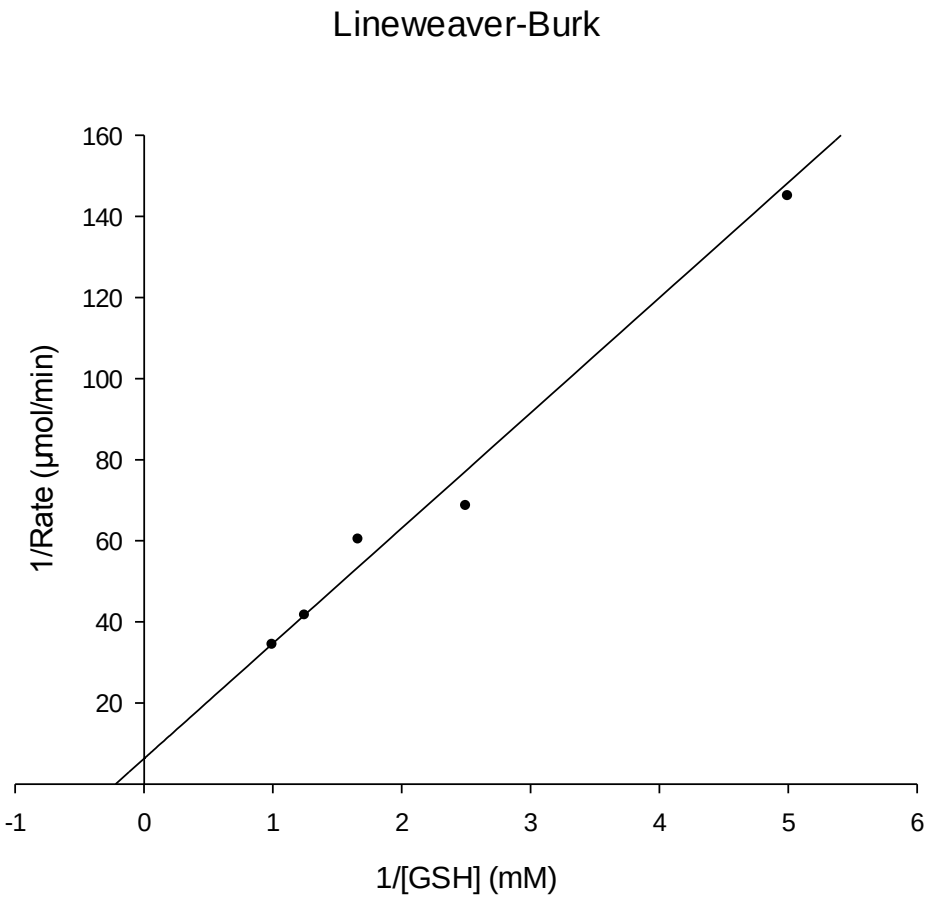
Vmax = 0.05058
Km = 0.9899

Kinetics of GST-P in the presence of *Euodia redlevi* (fruit&flower) ethanolic extract



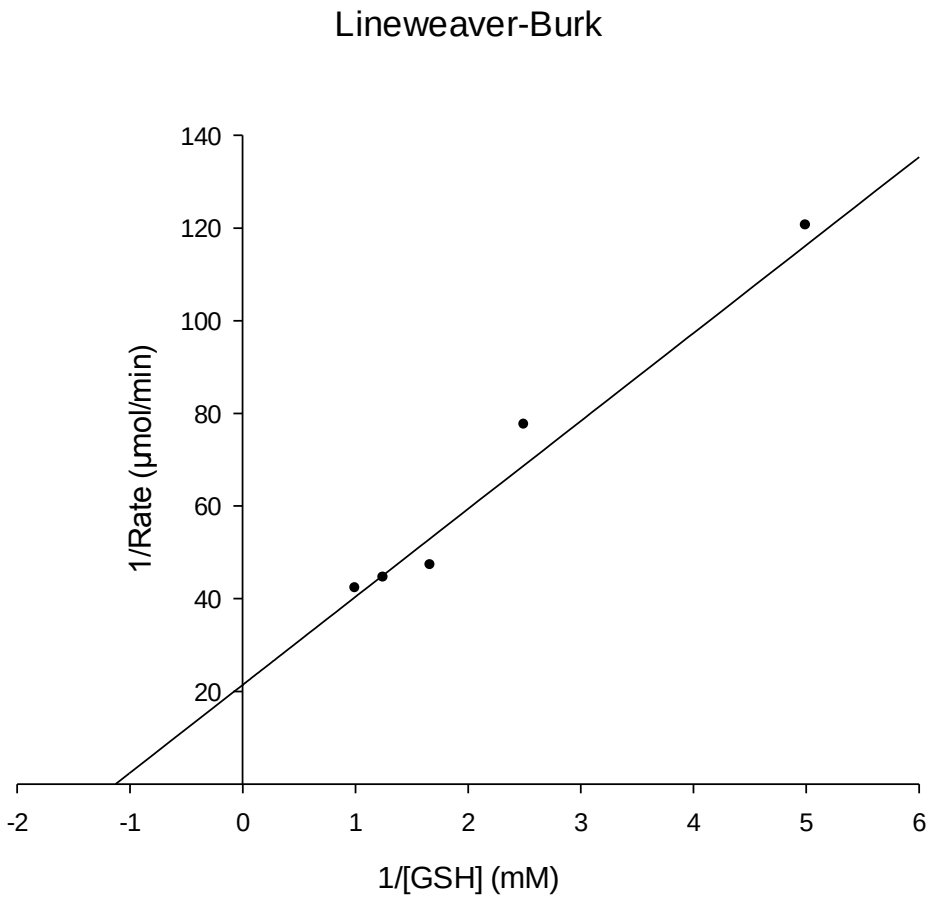
Vmax = 0.1498
Km = 3.01

Kinetics of GST-P in the presence of *Garcinia atroviridis* (branch) ethanolic extract



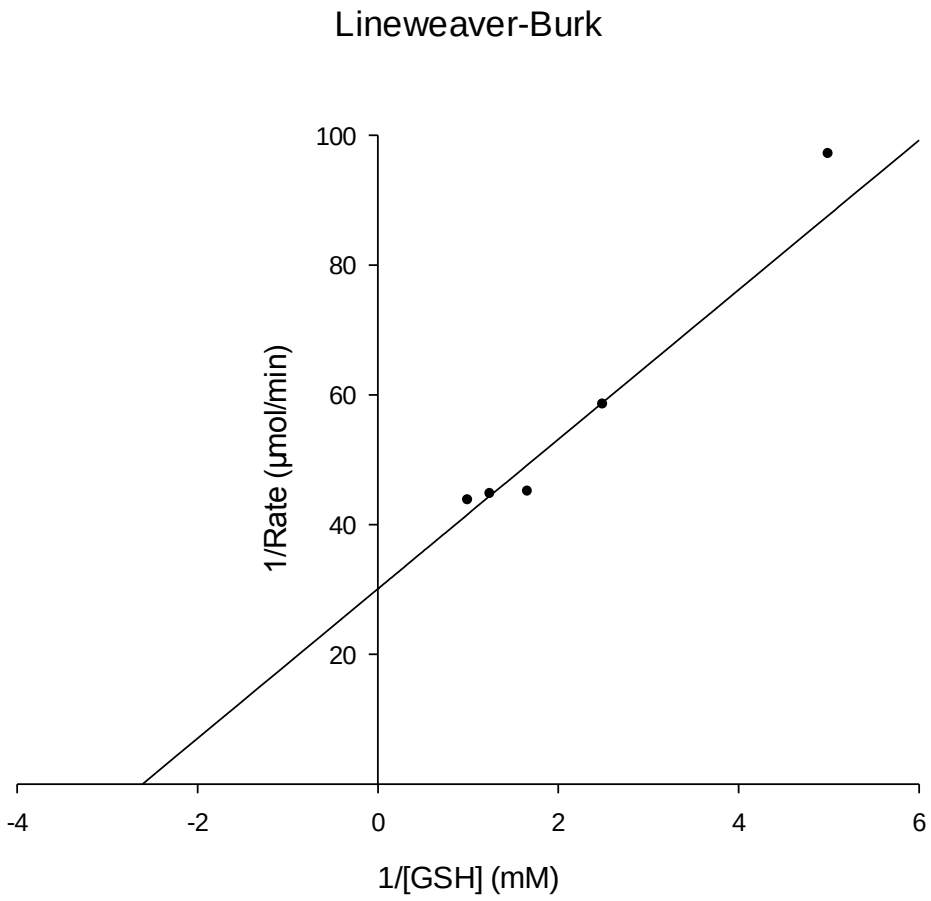
Vmax = 0.1588
Km = 4.51

Kinetics of GST-P in the presence of *Garcinia mangostana* (branch) ethanolic extract



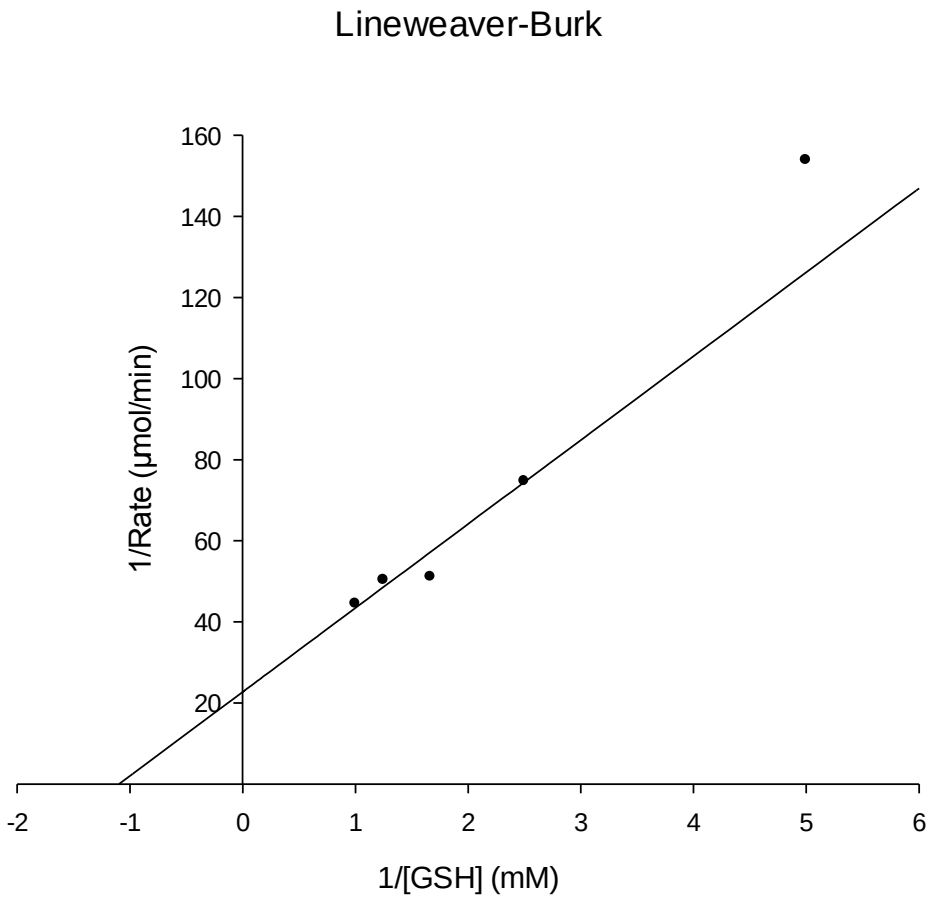
Vmax = 0.04668
Km = 0.886

Kinetics of GST-P in the presence of *Garcinia mangostana* (leaf) ethanolic extract



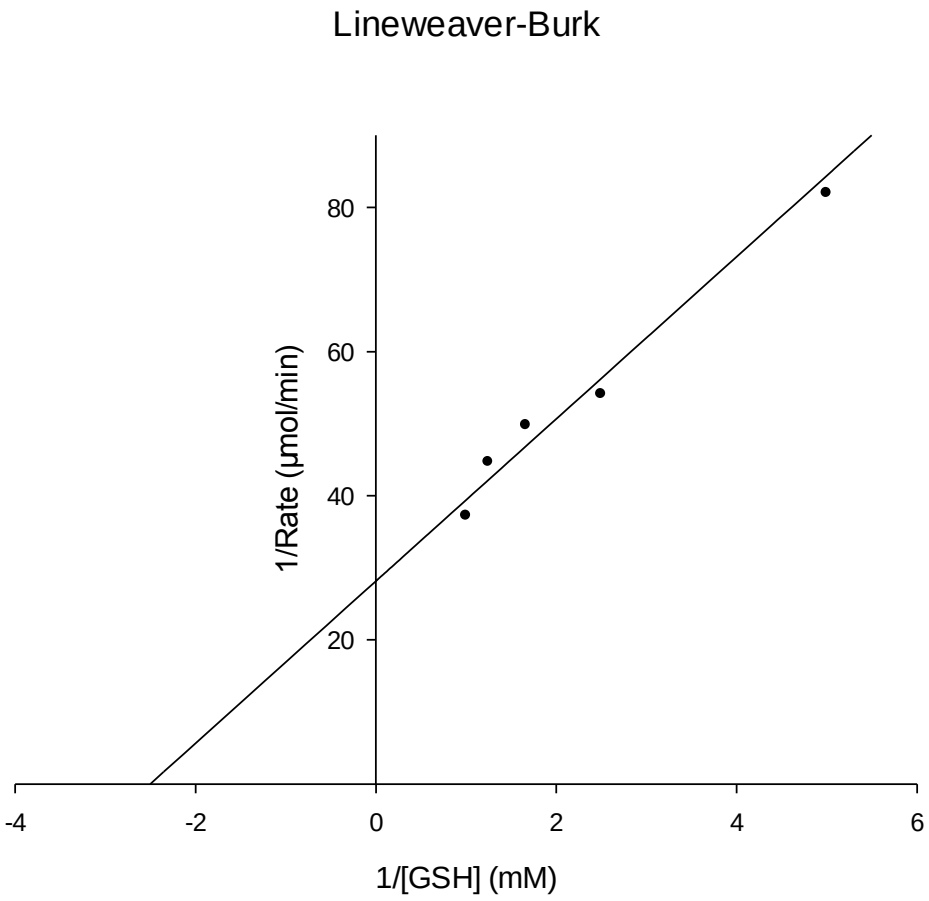
Vmax = 0.03322
Km = 0.3828

Kinetics of GST-P in the presence of *Hibiscus tiliaceus* (leaf) ethanolic extract



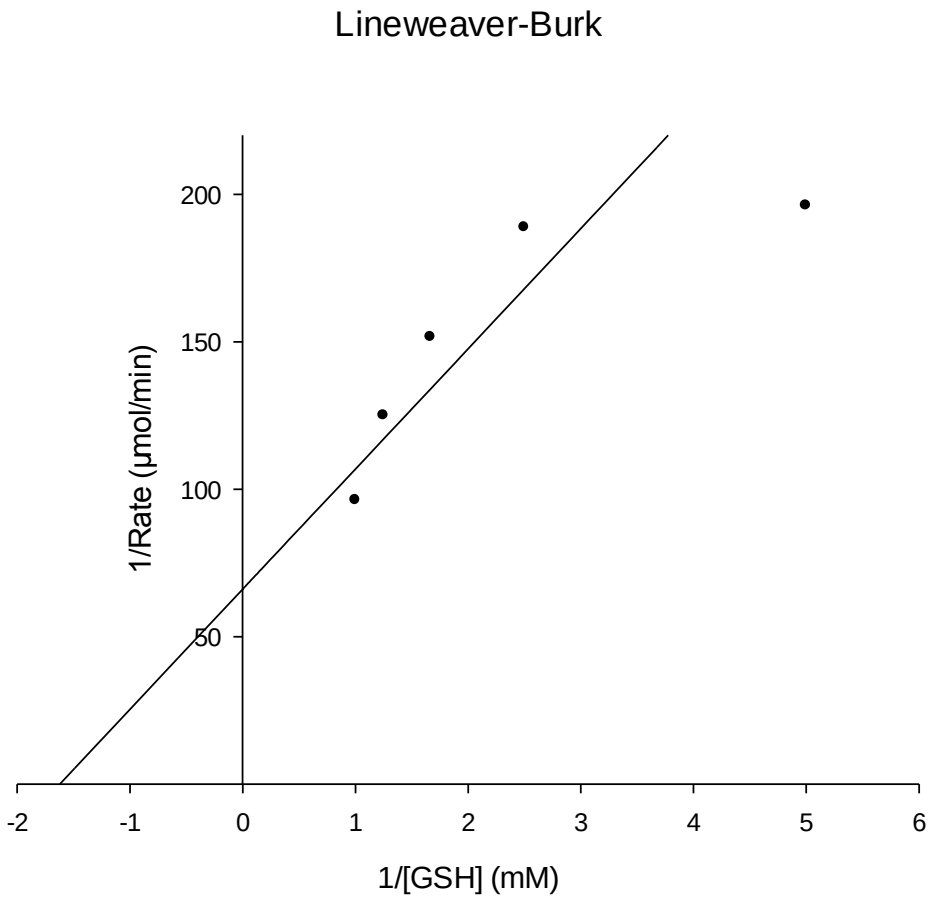
Vmax = 0.04397
Km = 0.9102

Kinetics of GST-P in the presence of *Lawsonia inermis* (branch) ethanolic extract



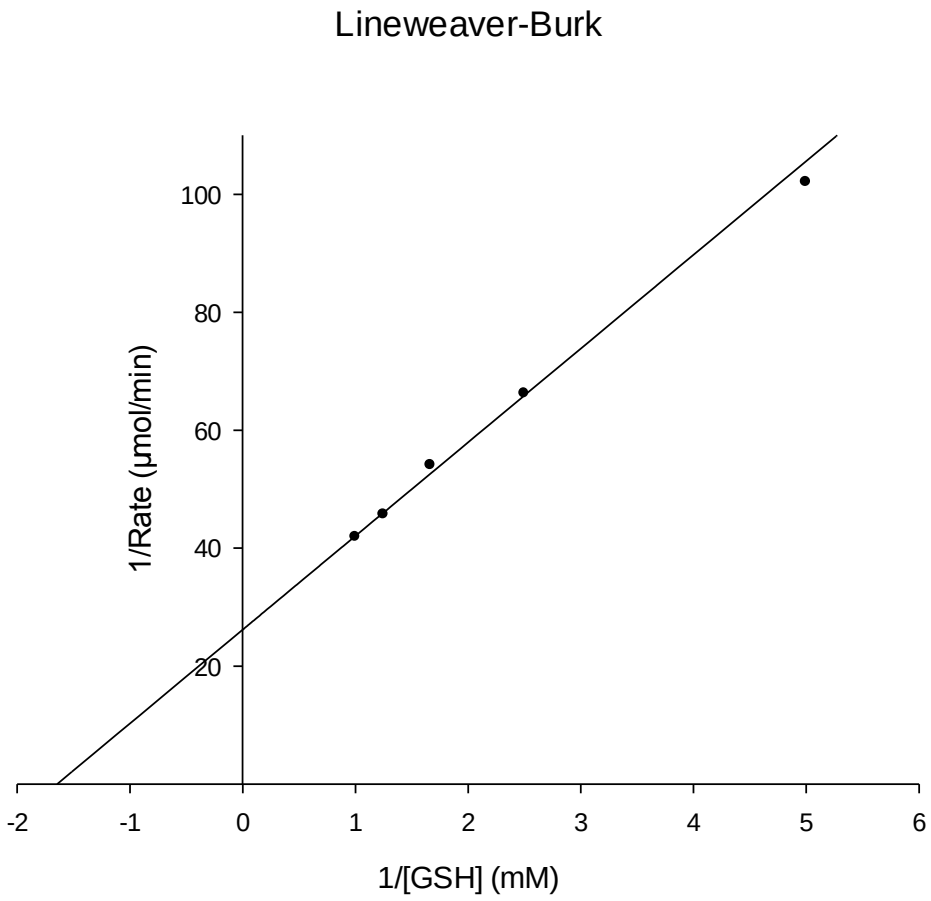
Vmax = 0.0355
Km = 0.3993

Kinetics of GST-P in the presence of *Leptospermum flavescens* (leaf) ethanolic extract



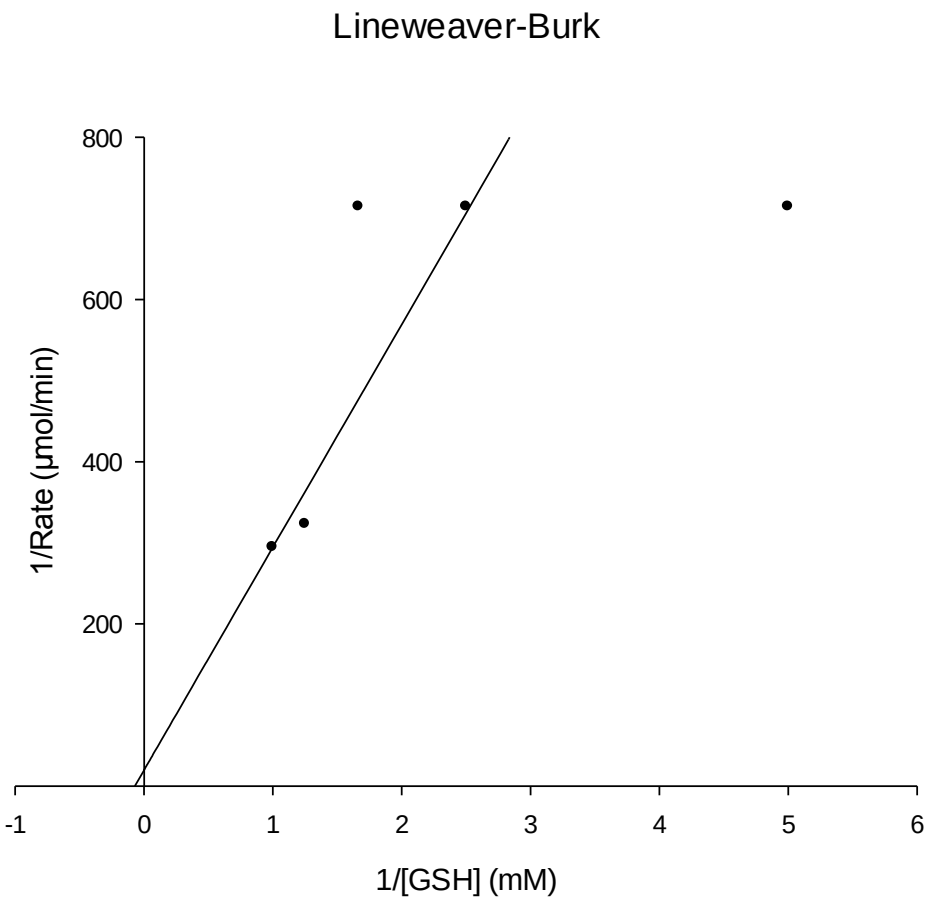
Vmax = 0.01512
Km = 0.6164

Kinetics of GST-P in the presence of *Oxalis barrelieri* (root) ethanolic extract



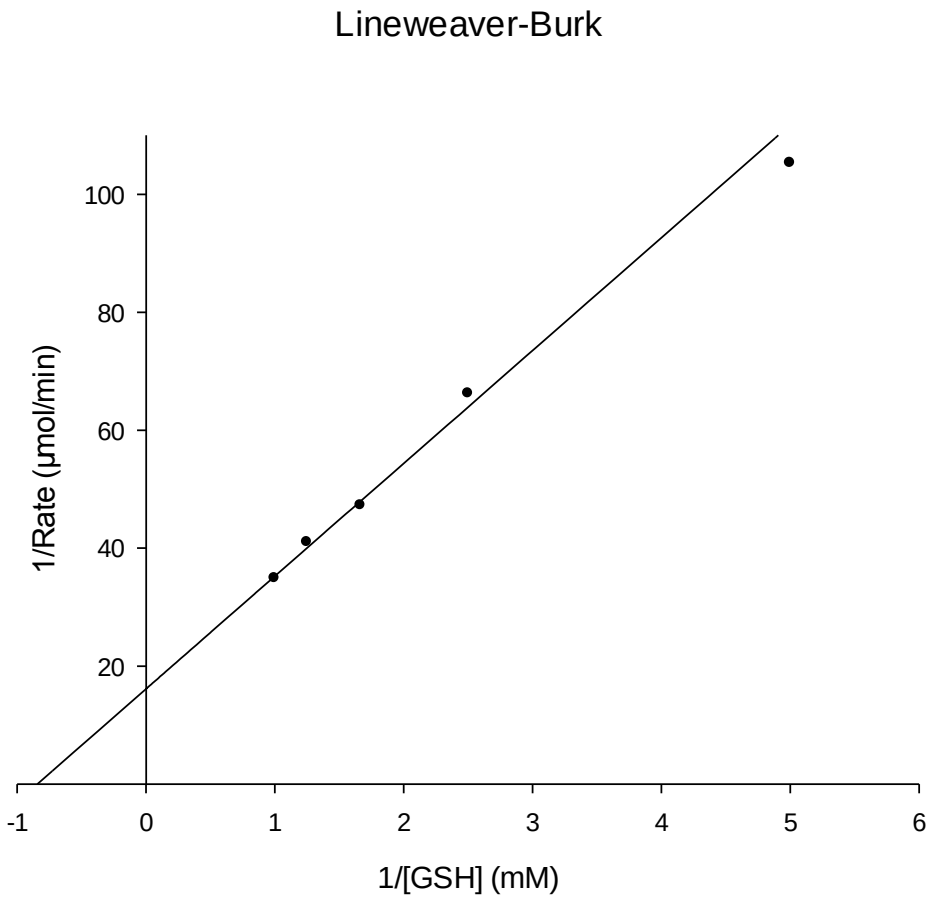
Vmax = 0.03819
Km = 0.607

Kinetics of GST-P in the presence of *Peltophorum pterocarpum* (leaf) ethanolic extract



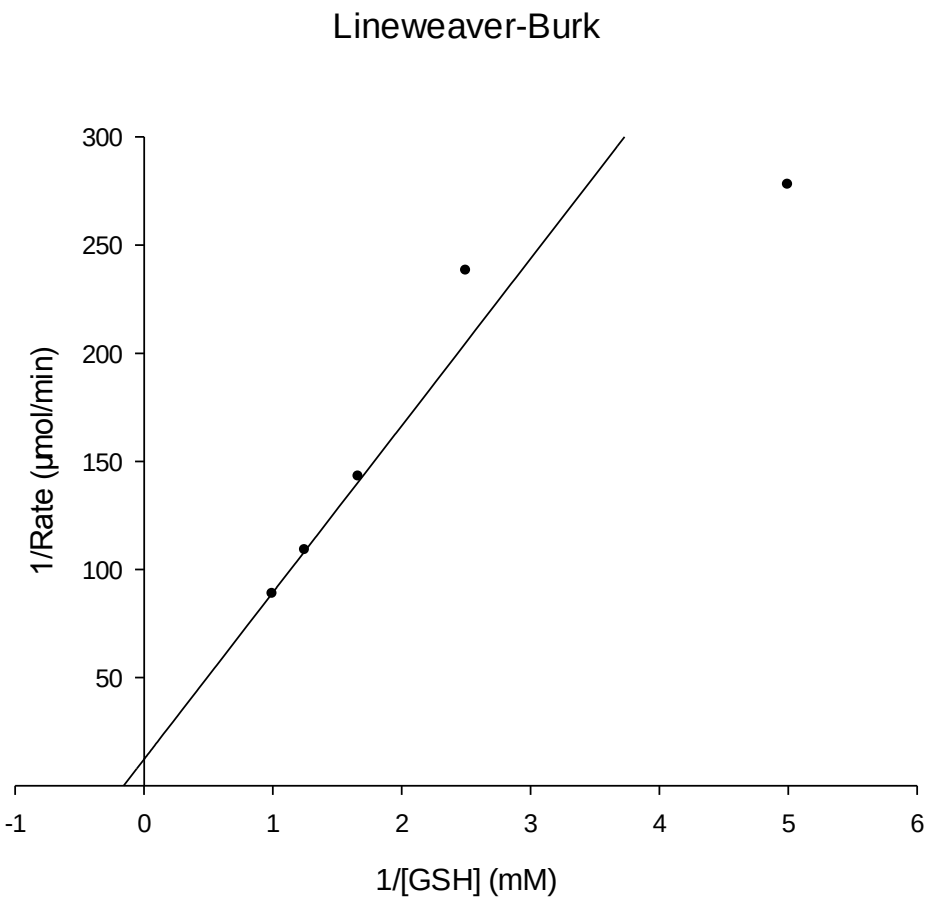
Vmax = 0.05029
Km = 13.82

Kinetics of GST-P in the presence of *Tetracera indica* (fruit) ethanolic extract



Vmax = 0.06193
Km = 1.18

Kinetics of GST-P in the presence of 50% methanol 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract



Vmax = 0.08156
Km = 6.29

TOXICITY EFFECTS OF SAMPLES ON MRC-5 CELLS

Figure 7.21 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride on MRC-5 cells. The drug inhibited MRC-5 cell proliferation in dose-dependent manner. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.82 ± 0.011 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

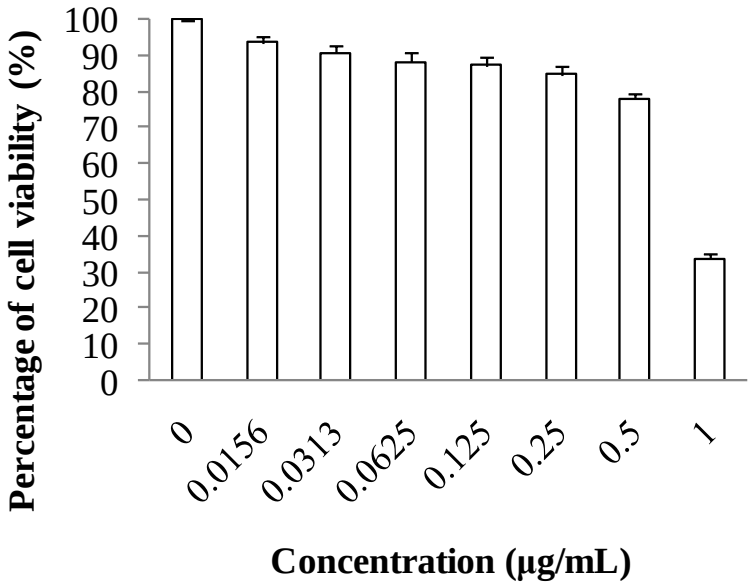


Figure 7.21 : Cytotoxicity effects of doxorubicin hydrochloride on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: doxorubicin hydrochloride

Normality Test (Shapiro-Wilk) Passed (P = 0.401)

Equal Variance Test: Passed (P = 0.972)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	33.653	1.345	0.776
0.5	3	0	78.071	1.222	0.705
0.25	3	0	84.929	2.394	1.382
0.125	3	0	87.314	2.187	1.262
0.0625	3	0	88.254	2.768	1.598
0.0313	3	0	90.941	2.261	1.305
0.0156	3	0	93.648	2.019	1.165
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8905.078	1272.154	331.931	<0.001
Residual	16	61.321	3.833		
Total	23	8966.399			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	66.347	41.507	<0.001	Yes
0 vs. 0.5	21.929	13.719	<0.001	Yes
0 vs. 0.25	15.071	9.428	<0.001	Yes
0 vs. 0.125	12.686	7.936	<0.001	Yes
0 vs. 0.0625	11.746	7.348	<0.001	Yes
0 vs. 0.0313	9.059	5.667	<0.001	Yes
0 vs. 0.0156	6.352	3.974	0.001	Yes

Figure 7.22 shows *in vitro* cytotoxicity effects of cisplatin on MRC-5 cells. The drug inhibited MRC-5 cell proliferation in dose-dependent manner. The IC_{50} value of cisplatin generated from the dose-response graph is 4.07 ± 0.04 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

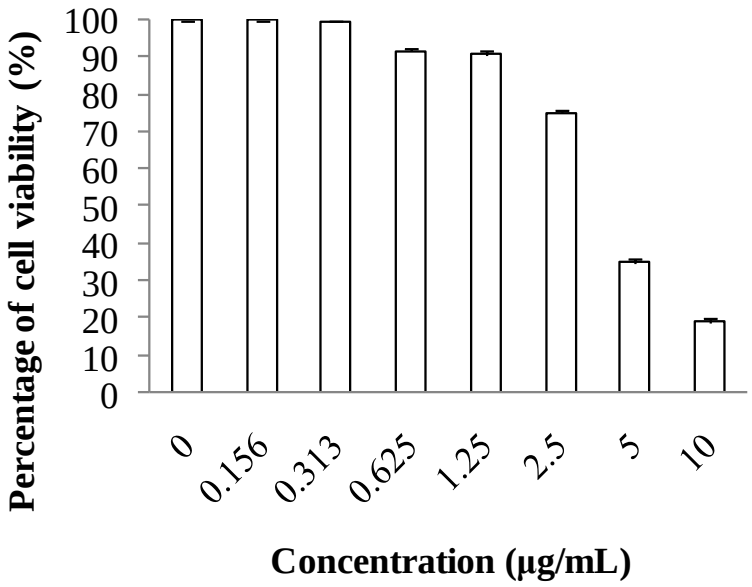


Figure 7.22 : Cytotoxicity effects of cisplatin on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: cisplatin

Normality Test (Shapiro-Wilk) Passed (P = 0.098)

Equal Variance Test: Passed (P = 0.582)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	19.025	1.146	0.661
5	3	0	35.055	0.789	0.456
2.5	3	0	75.305	0.686	0.396
1.25	3	0	90.811	1.037	0.599
.625	3	0	91.682	0.970	0.560
.313	3	0	99.868	0.178	0.103
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	21325.131	3046.447	5470.122	<0.001
Residual	16	8.911	0.557		
Total	23	21334.042			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	80.975	132.892	<0.001	Yes
0 vs. 5	64.945	106.584	<0.001	Yes
0 vs. 2.5	24.695	40.528	<0.001	Yes
0 vs. 1.25	9.189	15.080	<0.001	Yes
0 vs. .625	8.318	13.651	<0.001	Yes
0 vs. .313	0.132	0.217	0.971	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.23 shows *in vitro* cytotoxicity effects of ethacrynic acid on MRC-5 cells. The drug inhibited MRC-5 cell proliferation in dose-dependent manner. The IC_{50} value of ethacrynic acid generated from the dose-response graph is 22.71 ± 0.24 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

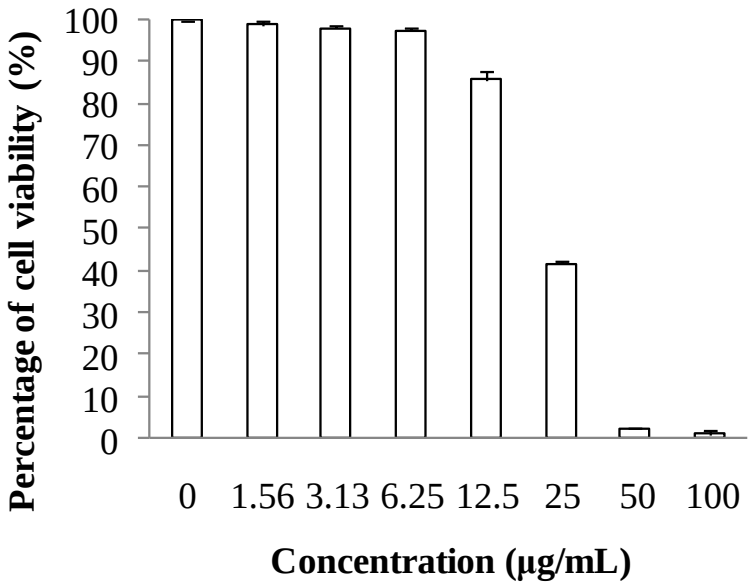


Figure 7.23 : Cytotoxicity effects of ethacrynic acid on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: ethacrynic acid

Normality Test (Shapiro-Wilk) Passed (P = 0.267)

Equal Variance Test: Passed (P = 0.901)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	1.247	1.076	0.621
50	3	0	2.515	0.362	0.209
25	3	0	41.938	0.687	0.396
12.5	3	0	85.996	1.659	0.958
6.25	3	0	97.566	0.552	0.319
3.13	3	0	98.091	0.770	0.444
1.56	3	0	98.976	0.948	0.547
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	40414.290	5773.470	7320.315	<0.001
Residual	16	12.619	0.789		
Total	23	40426.910			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	98.753	136.189	<0.001	Yes
0 vs. 50	97.485	134.440	<0.001	Yes
0 vs. 25	58.062	80.073	<0.001	Yes
0 vs. 12.5	14.004	19.313	<0.001	Yes
0 vs. 6.25	2.434	3.356	0.012	Yes
0 vs. 3.13	1.909	2.632	0.036	Yes
0 vs. 1.56	1.024	1.412	0.177	No

Figure 7.24 shows *in vitro* cytotoxicity effects of *Anacardium occidentale* (branch) ethanolic extract on MRC-5 cells. The IC_{50} value of *A. occidentale* (branch) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 0.466.

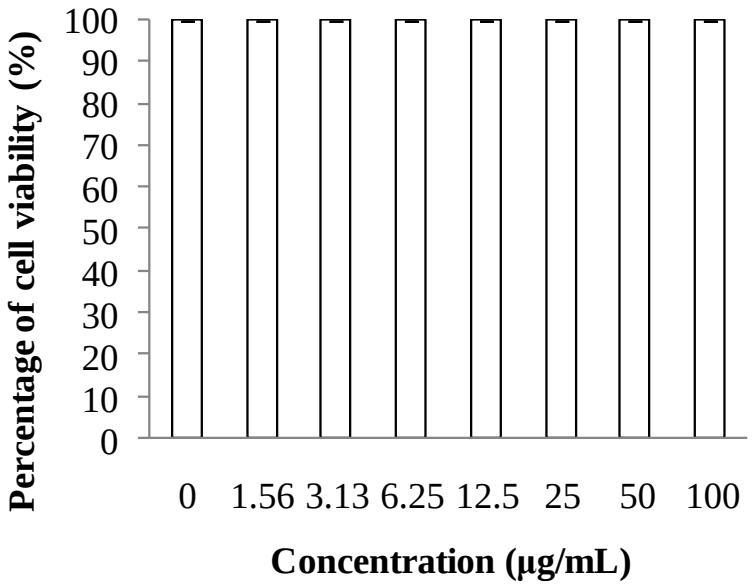


Figure 7.24 : Cytotoxicity effects of *A. occidentale* (branch) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Anacardium occidentale* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	99.970	0.0517	0.0299
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.00234	0.000335	1.000	0.466
Residual	16	0.00535	0.000335		
Total	23	0.00770			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.466).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 7.25 shows *in vitro* cytotoxicity effects of *Andrographis paniculata* (leaf) ethanolic extract on MRC-5 cells. The IC_{50} value of *A. paniculata* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

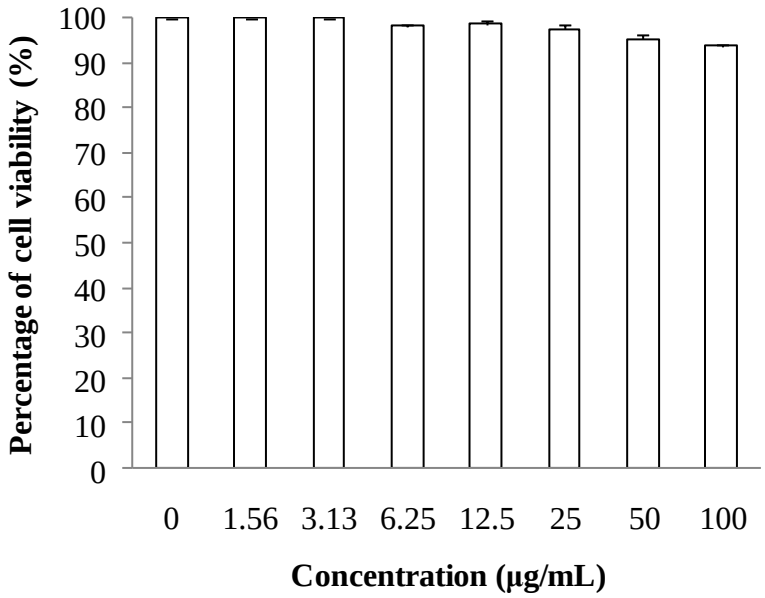


Figure 7.25 : Cytotoxicity effects of *A. paniculata* (leaf) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Andrographis paniculata* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.106)

Equal Variance Test: Passed (P = 0.784)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	93.859	0.498	0.287
50	3	0	95.473	1.084	0.626
25	3	0	97.512	1.110	0.641
12.5	3	0	98.642	0.924	0.533
6.25	3	0	98.337	0.339	0.196
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	108.888	15.555	34.339	<0.001
Residual	16	7.248	0.453		
Total	23	116.135			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	6.141	11.175	<0.001	Yes
0 vs. 50	4.527	8.238	<0.001	Yes
0 vs. 25	2.488	4.528	0.002	Yes
0 vs. 6.25	1.663	3.027	0.032	Yes
0 vs. 12.5	1.358	2.470	0.074	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No

Figure 7.26 shows *in vitro* cytotoxicity effects of *Cinnamomum zeylanicum* (branch) ethanolic extract on MRC-5 cells. The IC_{50} value of *C. zeylanicum* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

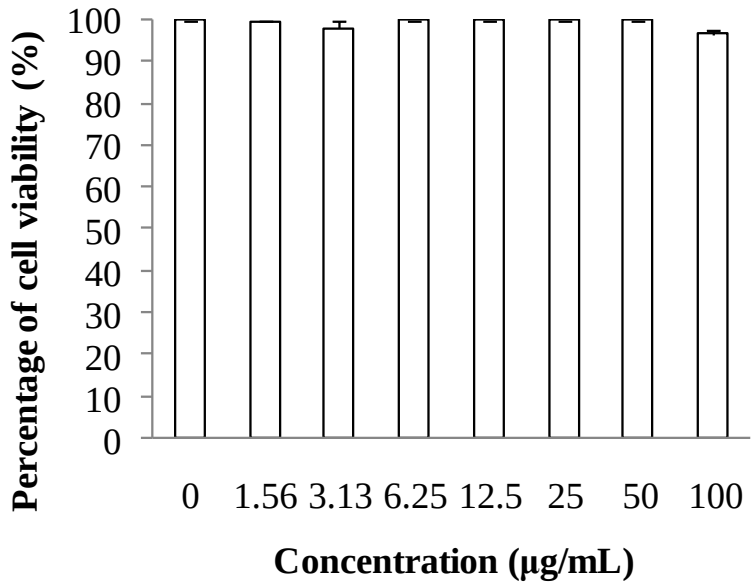


Figure 7.26 : Cytotoxicity effects of *C. zeylanicum* (branch) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Cinnamomum zeylanicum* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.256)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	96.916	0.960	0.554
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	99.946	0.0933	0.0538
3.13	3	0	98.106	1.699	0.981
1.56	3	0	99.736	0.458	0.264
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	28.989	4.141	8.230	<0.001
Residual	16	8.051	0.503		
Total	23	37.041			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.996

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	3.084	5.324	<0.001	Yes
0 vs. 3.13	1.894	3.270	0.029	Yes
0 vs. 1.56	0.264	0.456	0.995	No
0 vs. 6.25	0.0538	0.0930	1.000	No
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.27 shows *in vitro* cytotoxicity effects of *Euodia redlevi* (fruit&flower) ethanolic extract on MRC-5 cells. The IC_{50} value of *E. redleri* (fruit&flower) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = 0.005.

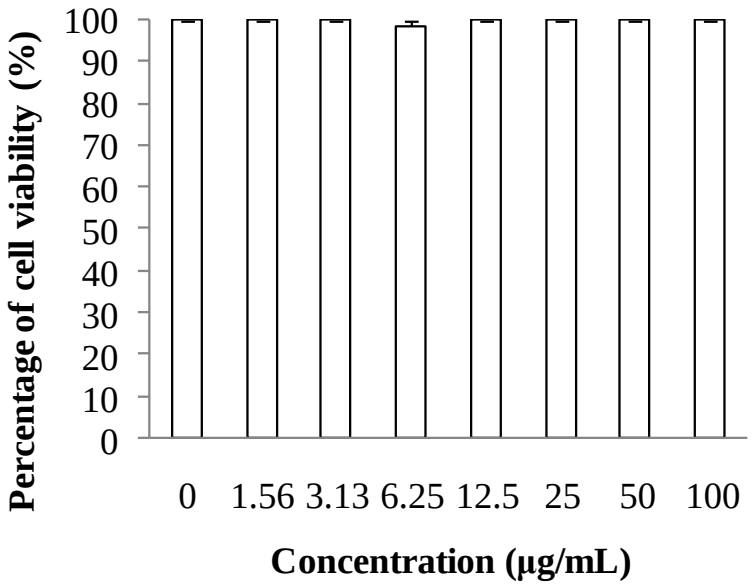


Figure 7.27 : Cytotoxicity effects of *E. redleri* (fruit&flower) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Euodia redlevi* (fruit&flower) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	98.805	0.956	0.552
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	3.747	0.535	4.682	0.005
Residual	16	1.829	0.114		
Total	23	5.576			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005).

Power of performed test with alpha = 0.050: 0.878

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 6.25	1.195	4.328	0.004	Yes
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 100	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.28 shows *in vitro* cytotoxicity effects of *Garcinia atroviridis* (branch) ethanolic extract on MRC-5 cells. The IC_{50} value of *G. atroviridis* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 0.533.

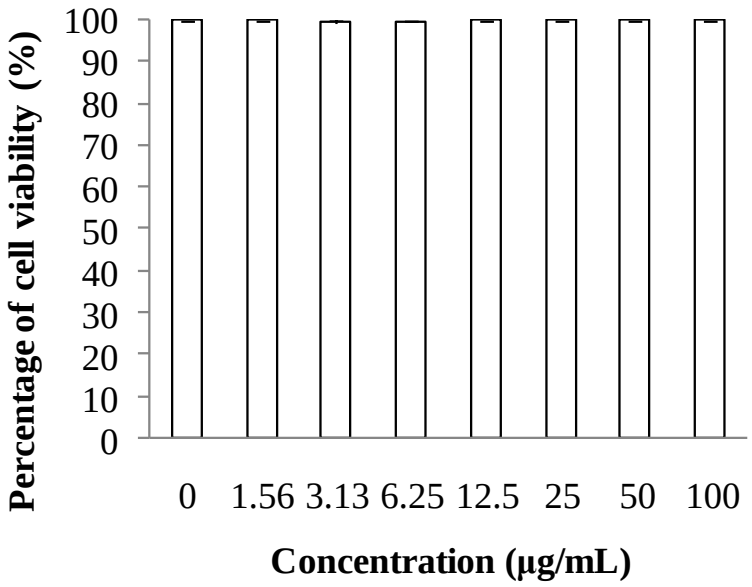


Figure 7.28 : Cytotoxicity effects of *G. atroviridis* (branch) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia atroviridis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	99.803	0.342	0.197
3.13	3	0	99.545	0.788	0.455
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.578	0.0826	0.896	0.533
Residual	16	1.475	0.0922		
Total	23	2.053			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.533).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 7.29 shows *in vitro* cytotoxicity effects of *Garcinia mangostana* (branch) ethanolic extract on MRC-5 cells. The IC_{50} value of *G. mangostana* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

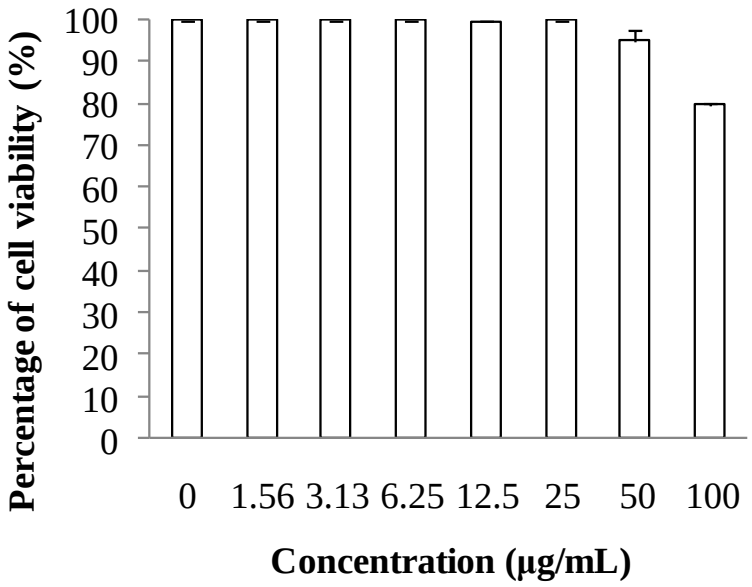


Figure 7.29 : Cytotoxicity effects of *G. mangostana* (branch) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.057)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	79.951	0.200	0.115
50	3	0	95.223	2.356	1.360
25	3	0	100.000	0.000	0.000
12.5	3	0	99.814	0.323	0.186
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	1039.832	148.547	208.648	<0.001
Residual	16	11.391	0.712		
Total	23	1051.223			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	20.049	29.101	<0.001	Yes
0 vs. 50	4.777	6.933	<0.001	Yes
0 vs. 12.5	0.186	0.270	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No

Figure 7.30 shows *in vitro* cytotoxicity effects of *Garcinia mangostana* (leaf) ethanolic extract on MRC-5 cells. The IC_{50} value of *G. mangostana* (leaf) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 1.0.

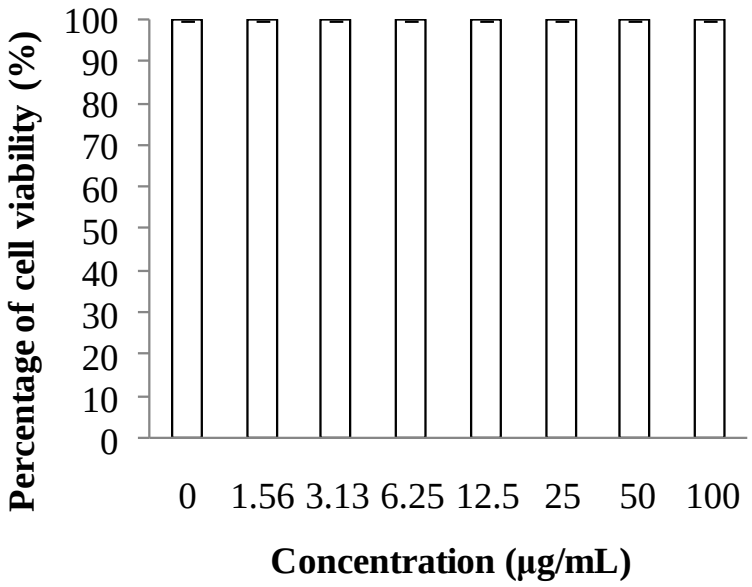


Figure 7.30 : Cytotoxicity effects of *G. mangostana* (leaf) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.000	0.000	1.000	1.000
Residual	16	0.000	0.000		
Total	23	0.000			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 1.000

Figure 7.31 shows *in vitro* cytotoxicity effects of *Hibiscus tiliaceus* (leaf) ethanolic extract on MRC-5 cells. The IC_{50} value of *H. tiliaceus* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 1.0.

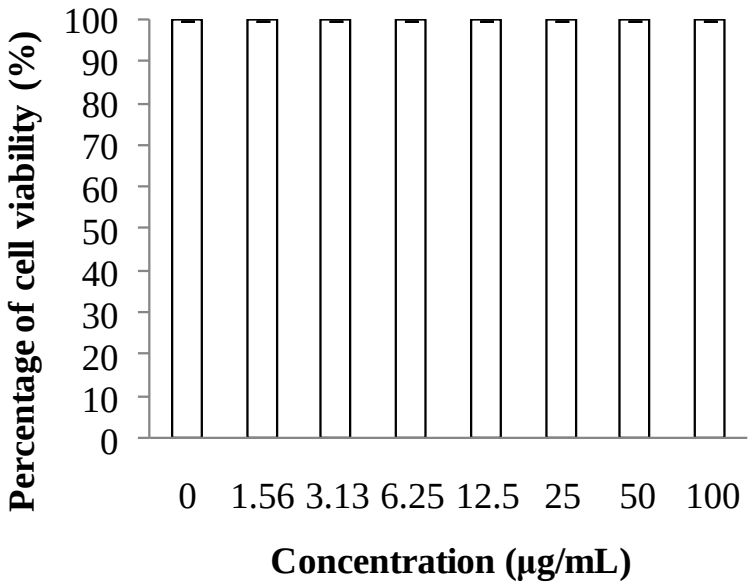


Figure 7.31 : Cytotoxicity effects of *H. tiliaceus* (leaf) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Hibiscus tiliaceus* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.000	0.000	1.000	1.000
Residual	16	0.000	0.000		
Total	23	0.000			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 1.000

Figure 7.32 shows *in vitro* cytotoxicity effects of *Lawsonia inermis* (branch) ethanolic extract on MRC-5 cells. The IC_{50} value of *L. inermis* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

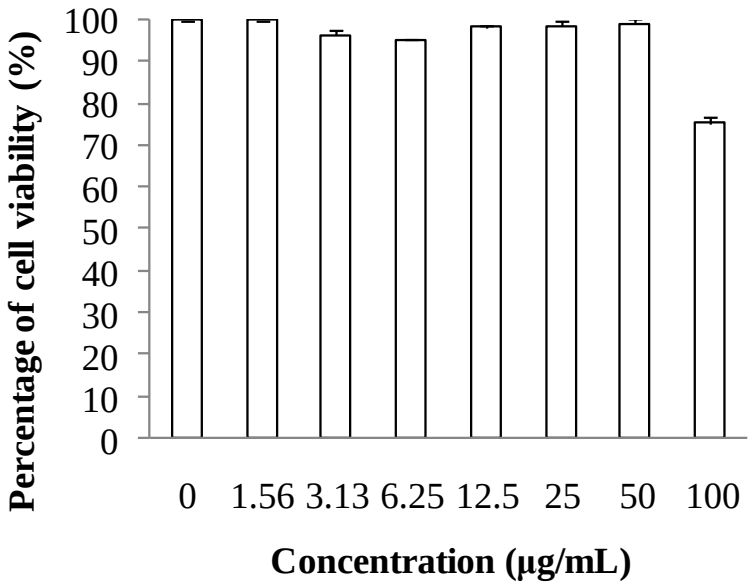


Figure 7.32 : Cytotoxicity effects of *L. inermis* (branch) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Lawsonia inermis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.433)

Equal Variance Test: Passed (P = 0.057)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	75.381	1.371	0.792
50	3	0	99.284	1.240	0.716
25	3	0	98.733	0.947	0.547
12.5	3	0	98.561	0.344	0.199
6.25	3	0	95.346	0.459	0.265
3.13	3	0	96.648	0.985	0.569
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	1442.246	206.035	293.546	<0.001
Residual	16	11.230	0.702		
Total	23	1453.476			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	24.619	35.990	<0.001	Yes
0 vs. 6.25	4.654	6.804	<0.001	Yes
0 vs. 3.13	3.352	4.900	<0.001	Yes
0 vs. 12.5	1.439	2.103	0.191	No
0 vs. 25	1.267	1.852	0.228	No
0 vs. 50	0.716	1.047	0.525	No
0 vs. 1.56	0.000	0.000	1.000	No

Figure 7.33 shows *in vitro* cytotoxicity effects of *Leptospermum flavescens* (leaf) ethanolic extract on MRC-5 cells. The IC_{50} value of *L. flavescens* (leaf) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 1.0.

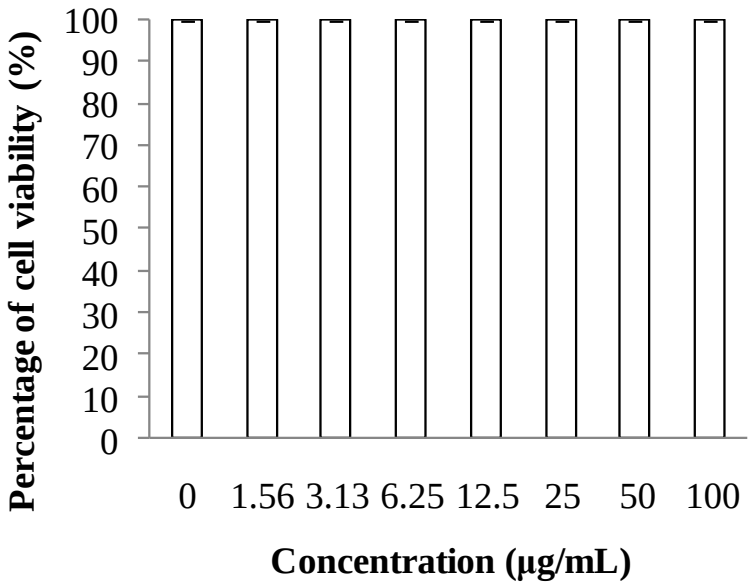


Figure 7.33 : Cytotoxicity effects of *L. flavescens* (leaf) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Leptospermum flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.000	0.000	1.000	1.000
Residual	16	0.000	0.000		
Total	23	0.000			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 1.000

Figure 7.34 shows *in vitro* cytotoxicity effects of *Oxalis barrelieri* (root) ethanolic extract on MRC-5 cells. The IC_{50} value of *O. barrelieri* (root) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

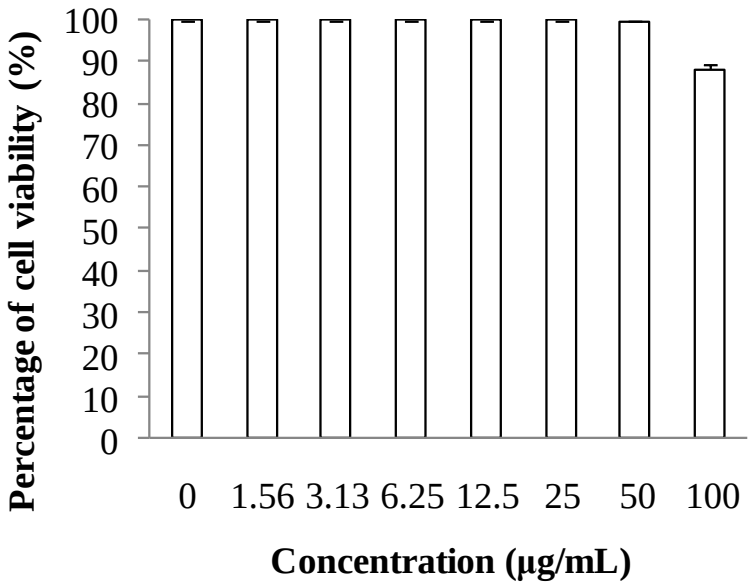


Figure 7.34 : Cytotoxicity effects of *O. barrelieri* (root) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Oxalis barrelieri* (root) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	88.344	1.264	0.730
50	3	0	99.763	0.411	0.237
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	354.740	50.677	229.541	<0.001
Residual	16	3.532	0.221		
Total	23	358.272			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	11.656	30.383	<0.001	Yes
0 vs. 50	0.237	0.618	0.991	No
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No

Figure 7.35 shows *in vitro* cytotoxicity effects of *Peltophorum pterocarpum* (leaf) ethanolic extract on MRC-5 cells. The IC_{50} value of *P. pterocarpum* (leaf) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

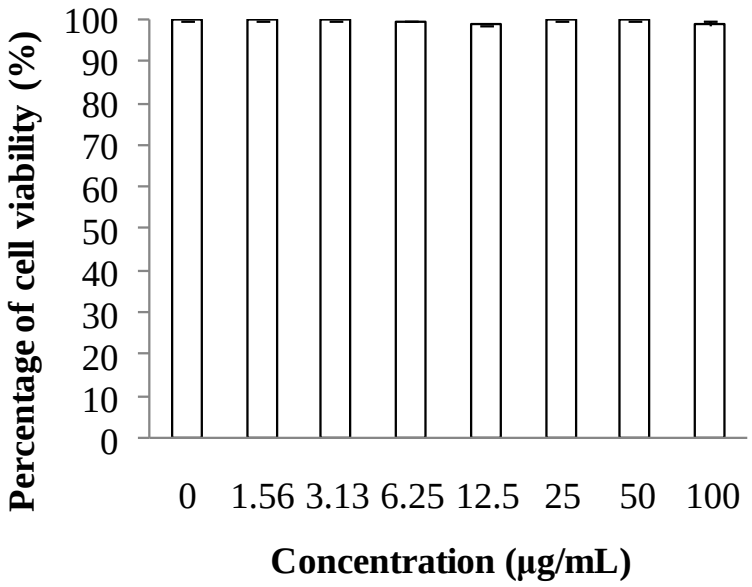


Figure 7.35 : Cytotoxicity effects of *P. pterocarpum* (leaf) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Peltophorum pterocarpum* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.414)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	99.045	0.830	0.479
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	98.899	0.132	0.0760
6.25	3	0	99.849	0.261	0.151
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	4.613	0.659	6.812	<0.001
Residual	16	1.548	0.0967		
Total	23	6.160			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.984

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 12.5	1.101	4.335	0.004	Yes
0 vs. 100	0.955	3.759	0.010	Yes
0 vs. 6.25	0.151	0.593	0.984	No
0 vs. 50	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No

Figure 7.36 shows *in vitro* cytotoxicity effects of *Tetracera indica* (fruit) ethanolic extract on MRC-5 cells. The IC_{50} value of *T. indica* (fruit) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

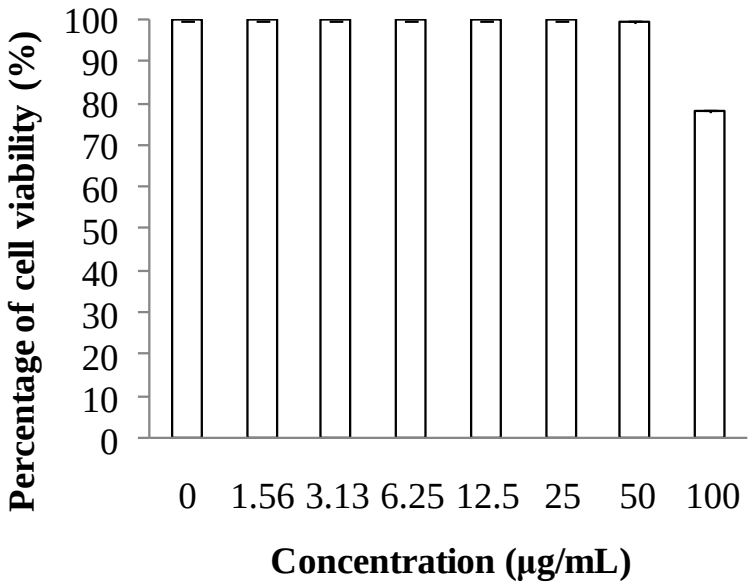


Figure 7.36 : Cytotoxicity effects of *T. indica* (fruit) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Tetracera indica* (fruit) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.820)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	78.083	0.321	0.185
50	3	0	99.549	0.399	0.230
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	1253.990	179.141	5469.757	<0.001
Residual	16	0.524	0.0328		
Total	23	1254.514			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	21.917	148.321	<0.001	Yes
0 vs. 50	0.451	3.055	0.045	Yes
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No

Figure 7.37 shows *in vitro* cytotoxicity effects of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on MRC-5 cells. The IC_{50} value of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = 0.001.

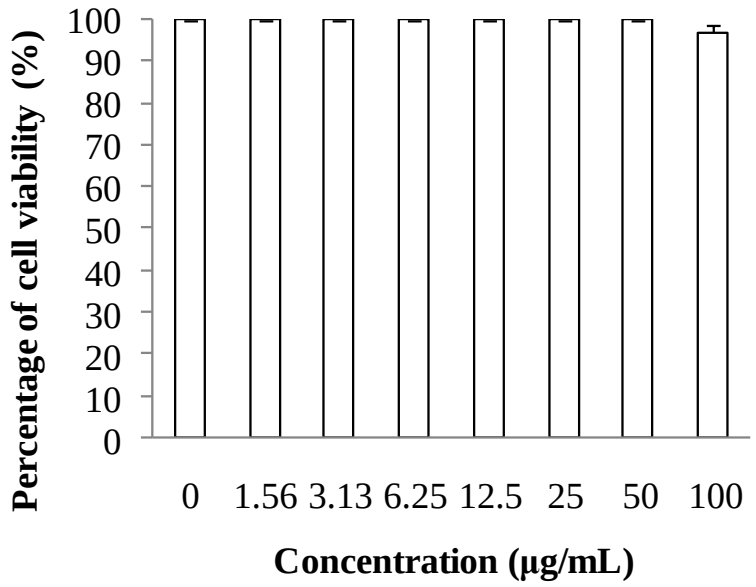


Figure 7.37 : Cytotoxicity effects of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on MRC-5 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	96.998	2.068	1.194
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	23.660	3.380	6.324	0.001
Residual	16	8.551	0.534		
Total	23	32.211			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001).

Power of performed test with alpha = 0.050: 0.973

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	3.002	5.030	<0.001	Yes
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

EFFECTS OF SAMPLES ON HT-29 CELL PROLIFERATION

Figure 7.38 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride on HT-29 cells. The drug inhibited HT-29 cell proliferation in dose-dependent manner. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.79 ± 0.046 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

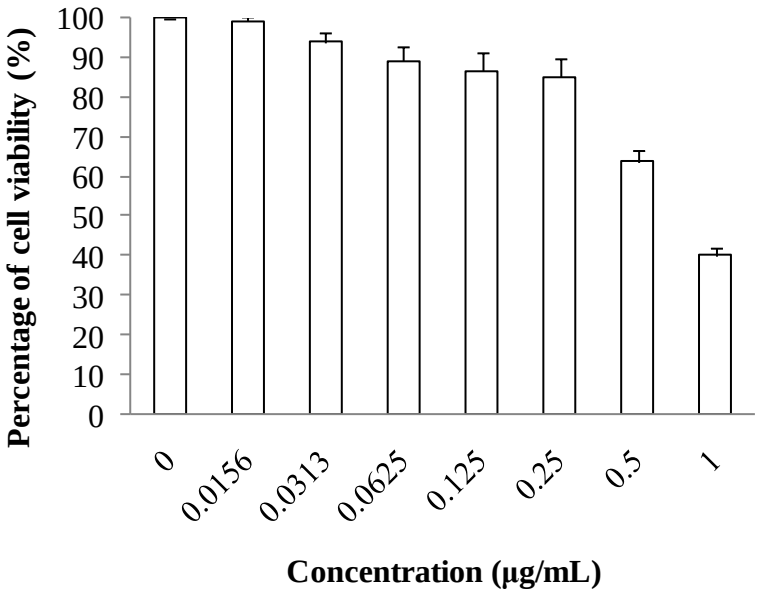


Figure 7.38 : Cytotoxicity effects of doxorubicin hydrochloride on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: Doxorubicin hydrochloride

Normality Test (Shapiro-Wilk) Passed (P = 0.338)

Equal Variance Test: Passed (P = 0.757)

Group Name	N	Missing	Mean	Std Dev	SEM
0	3	0	100.000	0.000	0.000
0.0156	3	0	99.354	1.118	0.646
0.0313	3	0	93.979	2.372	1.369
0.0625	3	0	89.105	3.780	2.182
0.125	3	0	86.716	4.519	2.609
0.25	3	0	85.241	4.842	2.796
0.5	3	0	63.752	2.811	1.623
1	3	0	40.025	1.717	0.991

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8838.523	1262.646	133.118	<0.001
Residual	16	151.763	9.485		
Total	23	8990.286			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	59.975	23.850	<0.001	Yes
0 vs. 0.5	36.248	14.415	<0.001	Yes
0 vs. 0.25	14.759	5.869	<0.001	Yes
0 vs. 0.125	13.284	5.283	<0.001	Yes
0 vs. 0.0625	10.895	4.333	0.002	Yes
0 vs. 0.0313	6.021	2.395	0.058	No
0 vs. 0.0156	0.646	0.257	0.801	No

Figure 7.39 shows *in vitro* cytotoxicity effects of cisplatin on HT-29 cells. The drug inhibited HT-29 cell proliferation in dose-dependent manner. The IC_{50} value of cisplatin generated from the dose-response graph is 9.49 ± 0.13 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

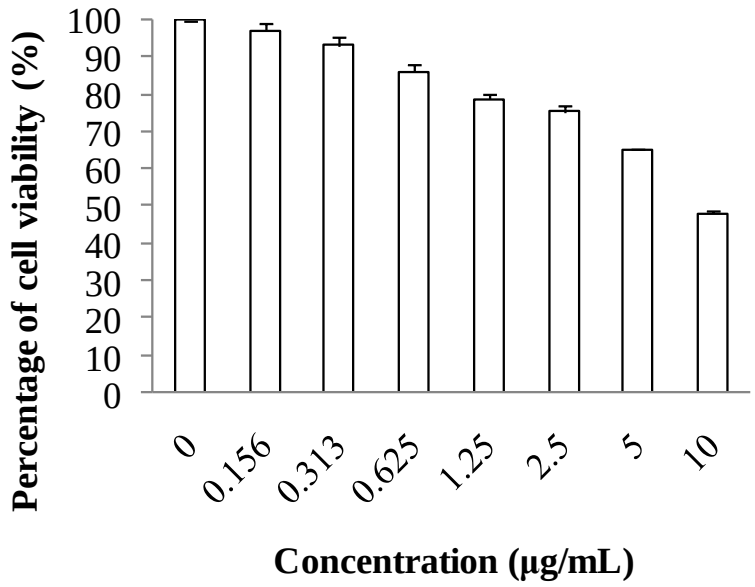


Figure 7.39 : Cytotoxicity effects of cisplatin on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: Cisplatin

Normality Test (Shapiro-Wilk) Passed (P = 0.620)

Equal Variance Test: Passed (P = 0.706)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	48.203	0.499	0.288
5	3	0	65.538	0.236	0.136
2.5	3	0	75.463	1.895	1.094
1.25	3	0	79.041	1.524	0.880
.625	3	0	86.214	2.302	1.329
.313	3	0	93.391	1.947	1.124
.156	3	0	97.230	1.899	1.097
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	6459.801	922.829	390.245	<0.001
Residual	16	37.836	2.365		
Total	23	6497.637			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	51.797	41.253	<0.001	Yes
0 vs. 5	34.462	27.447	<0.001	Yes
0 vs. 2.5	24.537	19.542	<0.001	Yes
0 vs. 1.25	20.959	16.693	<0.001	Yes
0 vs. .625	13.786	10.979	<0.001	Yes
0 vs. .313	6.609	5.264	<0.001	Yes
0 vs. .156	2.770	2.206	0.042	Yes

Figure 7.40 shows *in vitro* cytotoxicity effects of ethacrynic acid on HT-29 cells. The drug inhibited HT-29 cell proliferation in dose-dependent manner. The IC_{50} value of ethacrynic acid generated from the dose-response graph is 19.0 ± 0.26 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

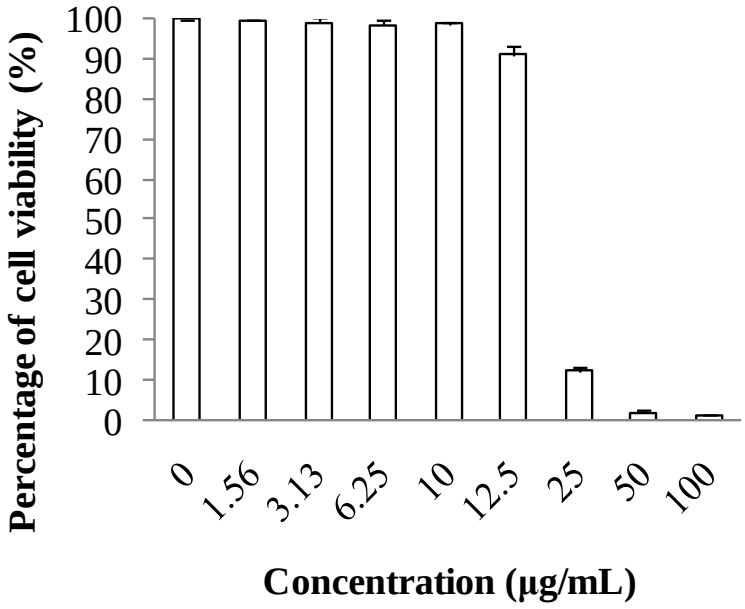


Figure 7.40 : Cytotoxicity effects of ethacrynic acid on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: Ethacrynic acid

Normality Test (Shapiro-Wilk) Passed (P = 0.179)

Equal Variance Test: Passed (P = 0.601)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	1.238	0.0982	0.0567
50	3	0	1.873	0.322	0.186
25	3	0	12.030	1.326	0.766
12.5	3	0	91.141	2.098	1.211
10	3	0	98.895	0.451	0.260
6.25	3	0	98.452	1.341	0.774
3.13	3	0	99.259	1.286	0.743
1.56	3	0	99.823	0.306	0.177
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	8	52152.764	6519.096	5854.124	<0.001
Residual	18	20.045	1.114		
Total	26	52172.809			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	98.762	114.624	<0.001	Yes
0 vs. 50	98.127	113.886	<0.001	Yes
0 vs. 25	87.970	102.099	<0.001	Yes
0 vs. 12.5	8.859	10.282	<0.001	Yes
0 vs. 6.25	1.548	1.797	0.312	No
0 vs. 10	1.105	1.283	0.518	No
0 vs. 3.13	0.741	0.860	0.642	No
0 vs. 1.56	0.177	0.205	0.840	No

Figure 7.41 shows *in vitro* cytotoxicity effects of *Anacardium occidentale* (branch) ethanolic extract on HT-29 cells. The extract inhibited HT-29 cell proliferation in dose-dependent manner. The IC_{50} value of *A. occidentale* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

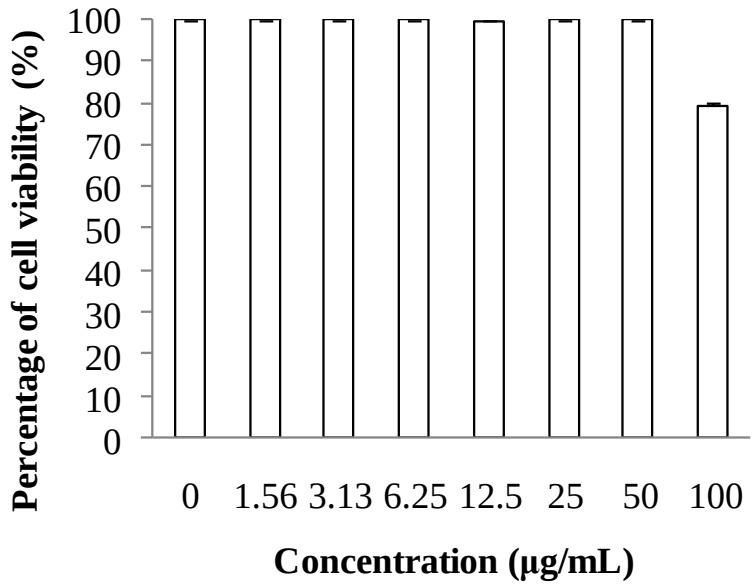


Figure 7.41 : Cytotoxicity effects of *A. occidentale* (branch) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Anacardium occidentale* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	79.505	0.526	0.303
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	99.917	0.144	0.0831
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	1101.362	157.337	4239.902	<0.001
Residual	16	0.594	0.0371		
Total	23	1101.955			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	20.495	130.303	<0.001	Yes
0 vs. 12.5	0.0831	0.528	0.996	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No

Figure 7.42 shows *in vitro* cytotoxicity effects of *Andrographis paniculata* (leaf) ethanolic extract on HT-29 cells. The extract inhibited HT-29 cell proliferation in dose-dependent manner. The IC_{50} value of *A. paniculata* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

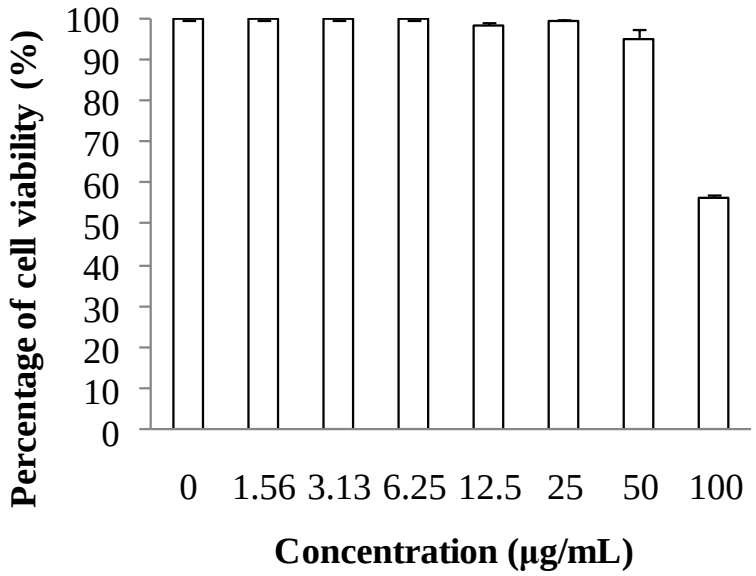


Figure 7.42 : Cytotoxicity effects of *A. paniculata* (leaf) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Andrographis paniculata* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	56.795	0.735	0.425
50	3	0	95.480	2.490	1.438
25	3	0	99.712	0.498	0.288
12.5	3	0	98.570	0.856	0.494
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	4751.001	678.714	703.169	<0.001
Residual	16	15.444	0.965		
Total	23	4766.444			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	43.205	53.860	<0.001	Yes
0 vs. 50	4.520	5.634	<0.001	Yes
0 vs. 12.5	1.430	1.783	0.388	No
0 vs. 25	0.288	0.358	0.994	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No

Figure 7.43 shows *in vitro* cytotoxicity effects of *Cinnamomum zeylanicum* (branch) ethanolic extract on HT-29 cells. The IC_{50} value of *C. zeylanicum* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

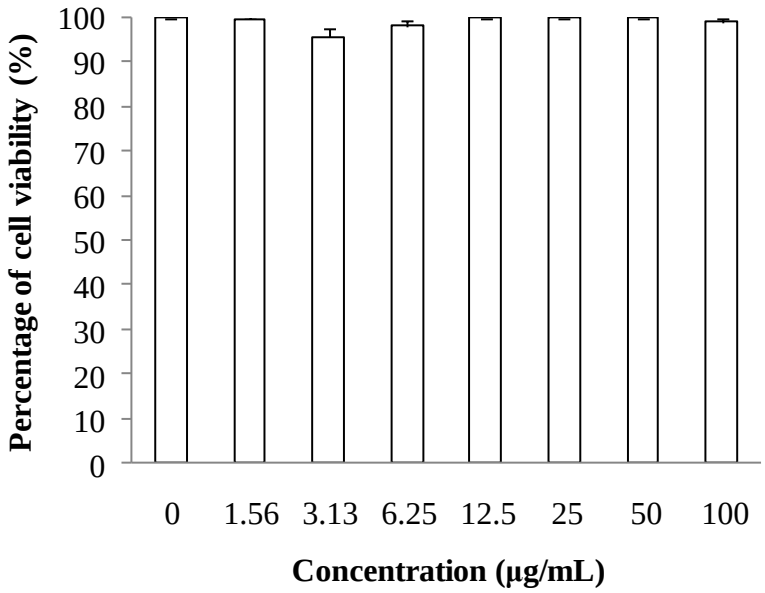


Figure 7.43 : Cytotoxicity effects of *C. zeylanicum* (branch) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Cinnamomum zeylanicum* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.516)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	99.063	0.922	0.532
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	98.169	1.158	0.668
3.13	3	0	95.755	1.968	1.136
1.56	3	0	99.742	0.447	0.258
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	47.133	6.733	8.603	<0.001
Residual	16	12.523	0.783		
Total	23	59.656			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.998

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 3.13	4.245	5.877	<0.001	Yes
0 vs. 6.25	1.831	2.535	0.125	No
0 vs. 100	0.937	1.297	0.698	No
0 vs. 1.56	0.258	0.357	0.994	No
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.44 shows *in vitro* cytotoxicity effects of *Euodia redlevi* (fruit&flower) ethanolic extract on HT-29 cells. The IC_{50} value of *E. redleri* (fruit&flower) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 0.099.

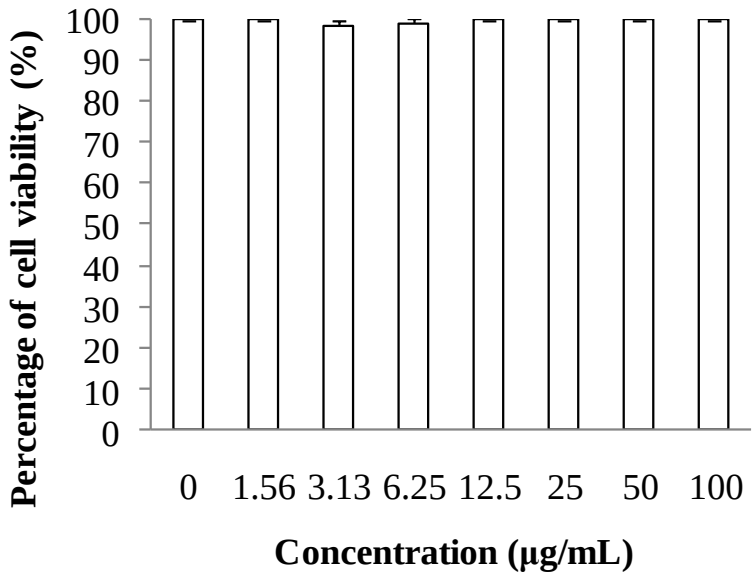


Figure 7.44 : Cytotoxicity effects of *E. redleri* (fruit&flower) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Euodia redlevi* (fruit&flower) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	99.372	1.088	0.628
3.13	3	0	98.625	1.297	0.749
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	5.350	0.764	2.133	0.099
Residual	16	5.734	0.358		
Total	23	11.084			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.099).

Power of performed test with alpha = 0.050: 0.335

The power of the performed test (0.335) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 7.45 shows *in vitro* cytotoxicity effects of *Garcinia atroviridis* (branch) ethanolic extract on HT-29 cells. The IC_{50} value of *G. atroviridis* (branch) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 0.346.

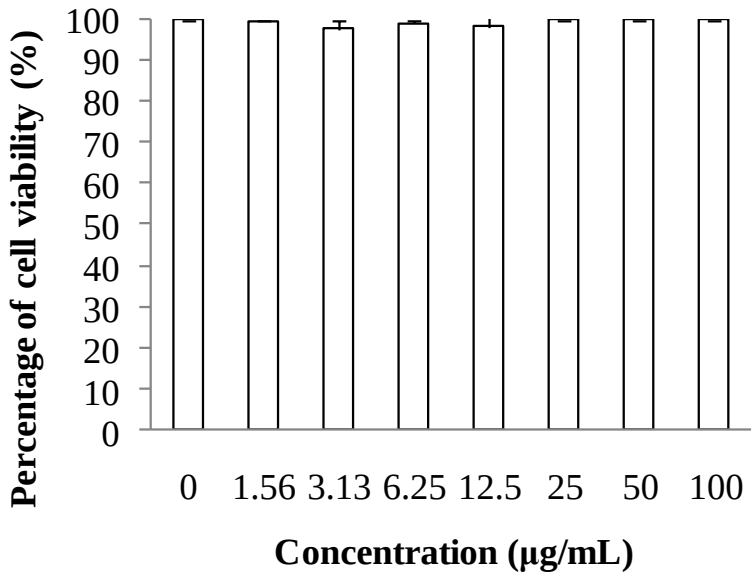


Figure 7.45 : Cytotoxicity effects of *G. atroviridis* (branch) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia atroviridis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	98.311	2.925	1.689
6.25	3	0	99.324	0.788	0.455
3.13	3	0	97.974	2.077	1.199
1.56	3	0	99.812	0.184	0.106
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	14.482	2.069	1.224	0.346
Residual	16	27.053	1.691		
Total	23	41.534			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.346).

Power of performed test with alpha = 0.050: 0.093

The power of the performed test (0.093) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 7.46 shows *in vitro* cytotoxicity effects of *Garcinia mangostana* (branch) ethanolic extract on HT-29 cells. The IC_{50} value of *G. mangostana* (branch) ethanolic extract generated from the dose-response graph is >100 μg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 1.0.

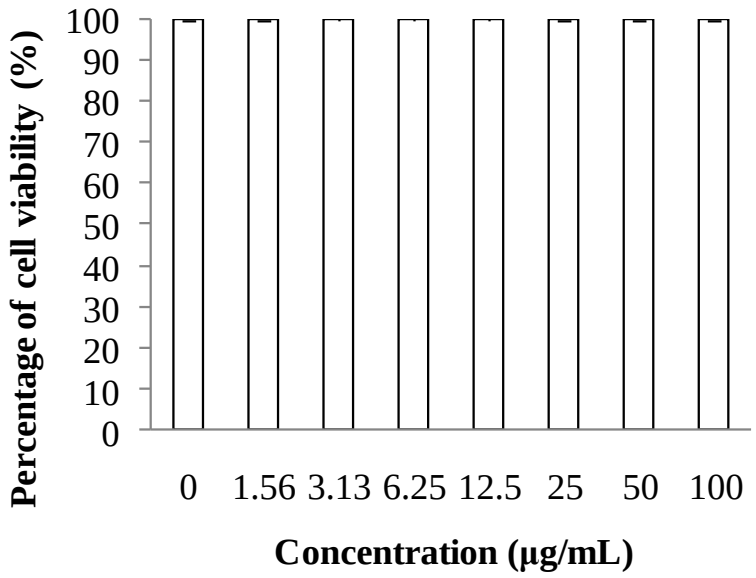


Figure 7.46 : Cytotoxicity effects of *G. mangostana* (branch) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.000	0.000	1.000	1.000
Residual	16	0.000	0.000		
Total	23	0.000			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 1.000

Figure 7.47 shows *in vitro* cytotoxicity effects of *Garcinia mangostana* (leaf) ethanolic extract on HT-29 cells. The IC_{50} value of *G. mangostana* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 1.0.

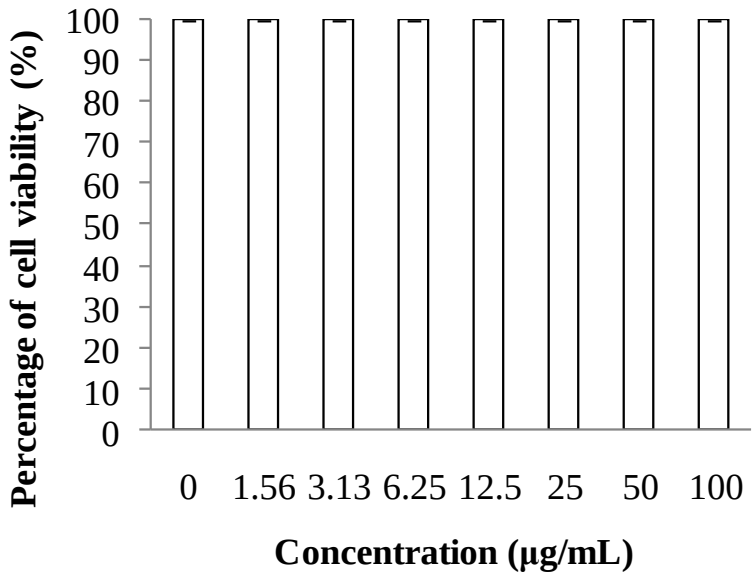


Figure 7.47 : Cytotoxicity effects of *G. mangostana* (leaf) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	0.000	0.000	1.000	1.000
Residual	16	0.000	0.000		
Total	23	0.000			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 1.000

Figure 7.48 shows *in vitro* cytotoxicity effects of *Hibiscus tiliaceus* (leaf) ethanolic extract on HT-29 cells. The IC_{50} value of *H. tiliaceus* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

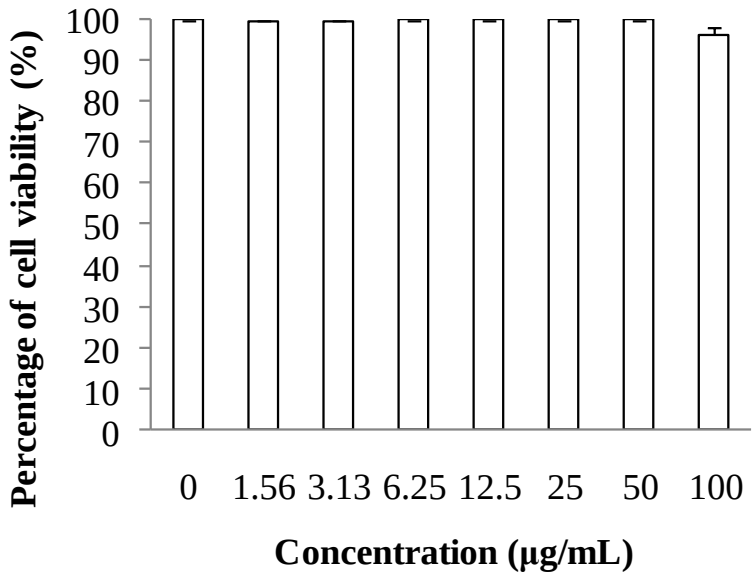


Figure 7.48 : Cytotoxicity effects of *H. tiliaceus* (leaf) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Hibiscus tiliaceus* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	96.442	1.552	0.896
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	99.733	0.462	0.267
1.56	3	0	99.771	0.397	0.229
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	32.185	4.598	13.231	<0.001
Residual	16	5.560	0.348		
Total	23	37.746			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	3.558	7.392	<0.001	Yes
0 vs. 3.13	0.267	0.554	0.995	No
0 vs. 1.56	0.229	0.476	0.994	No
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.49 shows *in vitro* cytotoxicity effects of *Lawsonia inermis* (branch) ethanolic extract on HT-29 cells. The IC_{50} value of *L. inermis* (branch) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates that there is not a statistically significant difference with P = 0.214.

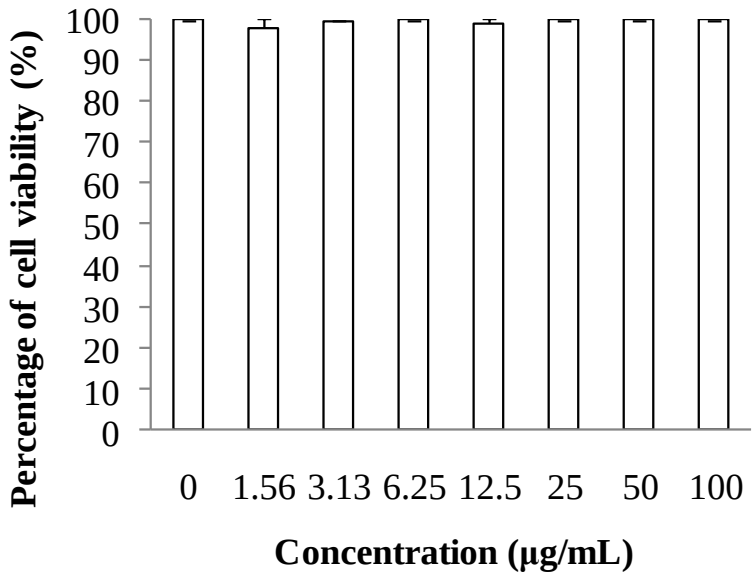


Figure 7.49 : Cytotoxicity effects of *L. inermis* (branch) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Lawsonia inermis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	99.353	1.120	0.647
6.25	3	0	100.000	0.000	0.000
3.13	3	0	99.793	0.359	0.207
1.56	3	0	98.020	2.448	1.413
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	10.136	1.448	1.571	0.214
Residual	16	14.749	0.922		
Total	23	24.885			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.214).

Power of performed test with alpha = 0.050: 0.176

The power of the performed test (0.176) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

Figure 7.50 shows *in vitro* cytotoxicity effects of *Leptospermum flavescens* (leaf) ethanolic extract on HT-29 cells. The IC_{50} value of *L. flavescens* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

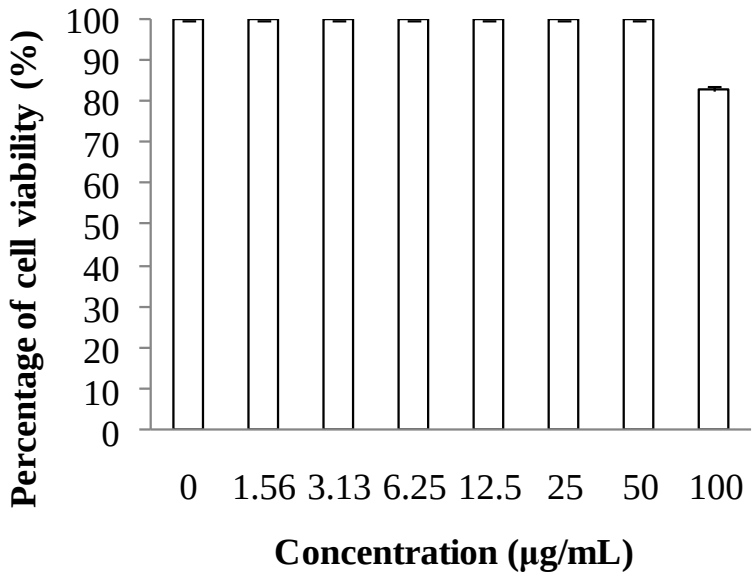


Figure 7.50 : Cytotoxicity effects of *L. flavescens* (leaf) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Leptospermum flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	82.897	1.163	0.671
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	767.877	109.697	648.759	<0.001
Residual	16	2.705	0.169		
Total	23	770.582			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	17.103	50.941	<0.001	Yes
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.51 shows *in vitro* cytotoxicity effects of *Oxalis barrelieri* (root) ethanolic extract on HT-29 cells. The IC_{50} value of *O. barrelieri* (root) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = 0.002.

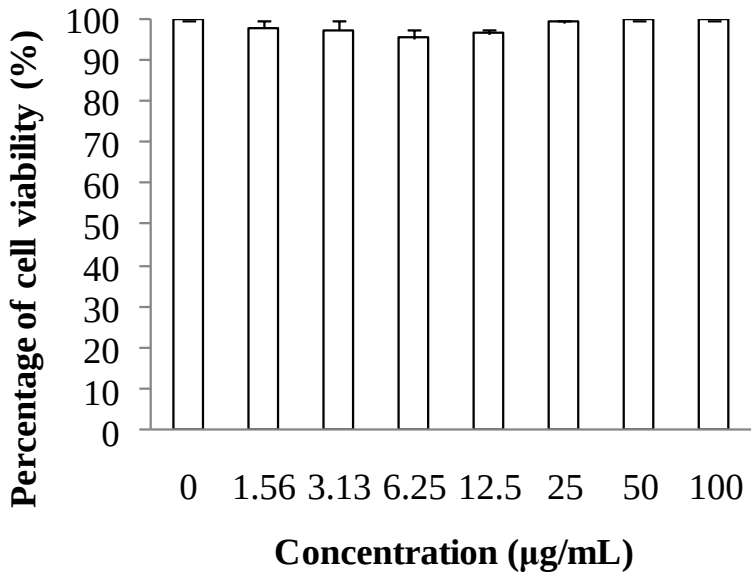


Figure 7.51 : Cytotoxicity effects of *O. barrelieri* (root) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Oxalis barrelieri* (root) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.628)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	99.544	0.465	0.268
12.5	3	0	96.711	1.218	0.703
6.25	3	0	95.783	1.914	1.105
3.13	3	0	97.623	2.096	1.210
1.56	3	0	98.172	1.489	0.860
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	57.885	8.269	5.523	0.002
Residual	16	23.954	1.497		
Total	23	81.839			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.002).

Power of performed test with alpha = 0.050: 0.942

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 6.25	4.217	4.221	0.005	Yes
0 vs. 12.5	3.289	3.292	0.027	Yes
0 vs. 3.13	2.377	2.380	0.142	No
0 vs. 1.56	1.828	1.830	0.302	No
0 vs. 25	0.456	0.457	0.959	No
0 vs. 100	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.52 shows *in vitro* cytotoxicity effects of *Peltophorum pterocarpum* (leaf) ethanolic extract on HT-29 cells. The IC_{50} value of *P. pterocarpum* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

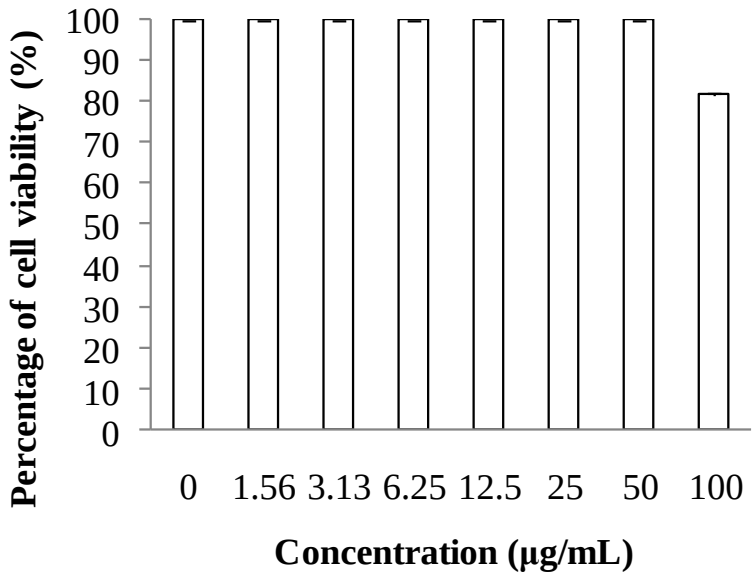


Figure 7.52 : Cytotoxicity effects of *P. pterocarpum* (leaf) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Peltophorum pterocarpum* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	81.929	0.442	0.255
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	100.000	0.000	0.000
6.25	3	0	100.000	0.000	0.000
3.13	3	0	100.000	0.000	0.000
1.56	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	857.192	122.456	5010.607	<0.001
Residual	16	0.391	0.0244		
Total	23	857.583			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 100	18.071	141.571	<0.001	Yes
0 vs. 12.5	0.000	0.000	1.000	No
0 vs. 1.56	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 6.25	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.53 shows *in vitro* cytotoxicity effects of *Tetracera indica* (fruit) ethanolic extract on HT-29 cells. The IC_{50} value of *T. indica* (fruit) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = 0.020.

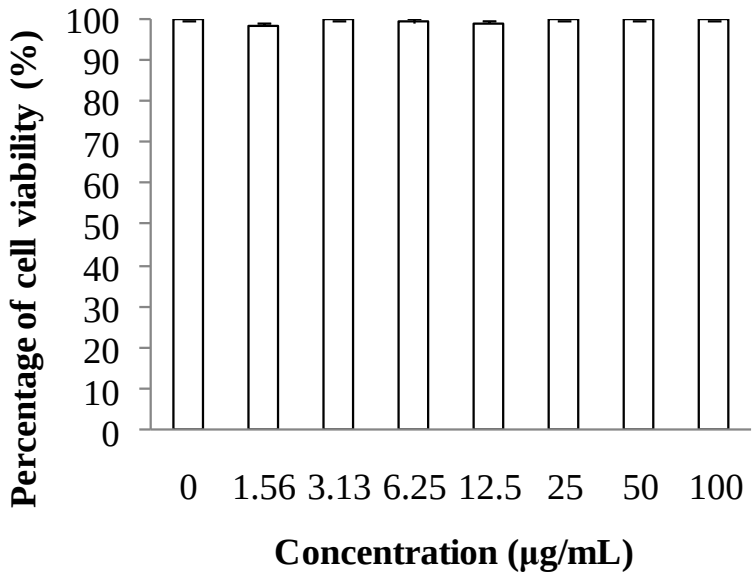


Figure 7.53 : Cytotoxicity effects of *T. indica* (fruit) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Tetracera indica* (fruit) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.280)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	99.354	0.622	0.359
6.25	3	0	99.543	0.792	0.457
3.13	3	0	100.000	0.000	0.000
1.56	3	0	98.732	0.726	0.419
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	4.594	0.656	3.406	0.020
Residual	16	3.083	0.193		
Total	23	7.677			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.020).

Power of performed test with alpha = 0.050: 0.677

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1.56	1.268	3.538	0.019	Yes
0 vs. 12.5	0.646	1.802	0.433	No
0 vs. 6.25	0.457	1.276	0.712	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 100	0.000	0.000	1.000	No
0 vs. 3.13	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

Figure 7.54 shows *in vitro* cytotoxicity effects of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on HT-29 cells. The IC_{50} value of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract generated from the dose-response graph is >100 µg/mL. Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

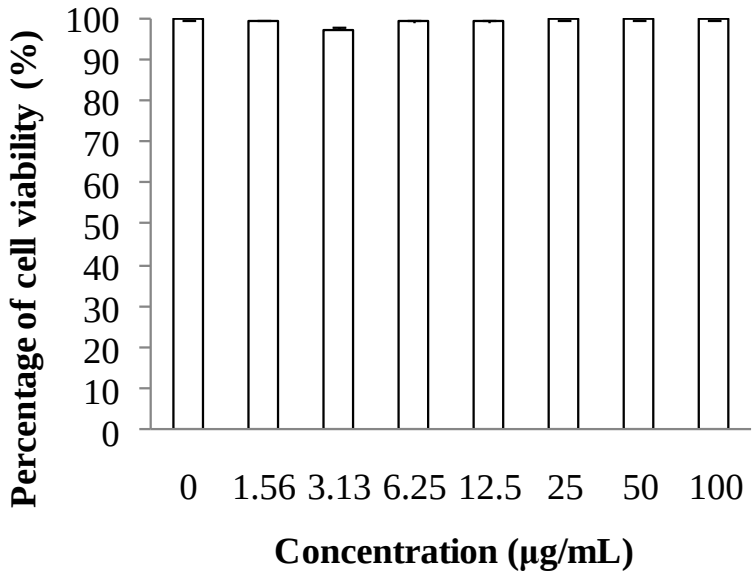


Figure 7.54 : Cytotoxicity effects of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.314)

Group Name	N	Missing	Mean	Std Dev	SEM
100	3	0	100.000	0.000	0.000
50	3	0	100.000	0.000	0.000
25	3	0	100.000	0.000	0.000
12.5	3	0	99.536	0.431	0.249
6.25	3	0	99.420	0.504	0.291
3.13	3	0	97.534	0.943	0.544
1.56	3	0	99.720	0.242	0.140
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	14.751	2.107	12.156	<0.001
Residual	16	2.774	0.173		
Total	23	17.524			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 3.13	2.466	7.255	<0.001	Yes
0 vs. 6.25	0.580	1.705	0.495	No
0 vs. 12.5	0.464	1.365	0.654	No
0 vs. 1.56	0.280	0.823	0.889	No
0 vs. 100	0.000	0.000	1.000	No
0 vs. 25	0.000	0.000	1.000	No
0 vs. 50	0.000	0.000	1.000	No

COMBINATION EFFECTS OF PLANT SAMPLES AND DOXORUBICIN HYDROCHLORIDE ON CYTOTOXICITY OF HT-29 CELLS

Figure 7.55 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with ethacrynic acid, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.19 ± 0.01 µg/mL. Concentration of ethacrynic acid was fixed at 10 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

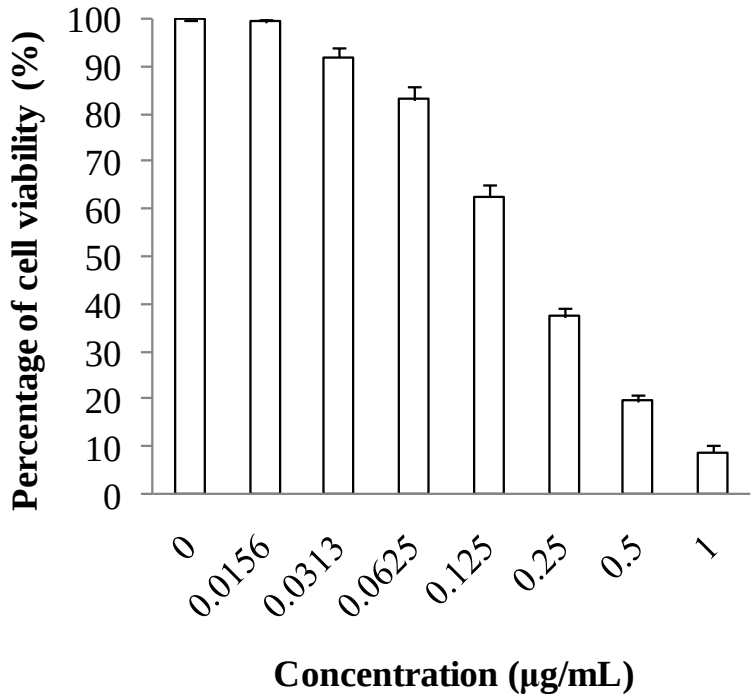


Figure 7.55 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with ethacrynic acid, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: Ethacrynic acid

Normality Test (Shapiro-Wilk) Passed (P = 0.672)

Equal Variance Test: Passed (P = 0.971)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	8.585	1.691	0.976
0.5	3	0	19.418	1.610	0.930
0.25	3	0	37.410	1.792	1.034
0.125	3	0	62.743	2.614	1.509
0.0625	3	0	83.269	2.493	1.439
0.0313	3	0	92.002	2.184	1.261
0.0156	3	0	99.594	0.703	0.406
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	28425.527	4060.790	1204.392	<0.001
Residual	16	53.946	3.372		
Total	23	28479.473			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	91.415	60.973	<0.001	Yes
0 vs. 0.5	80.582	53.748	<0.001	Yes
0 vs. 0.25	62.590	41.747	<0.001	Yes
0 vs. 0.125	37.257	24.850	<0.001	Yes
0 vs. 0.0625	16.731	11.160	<0.001	Yes
0 vs. 0.0313	7.998	5.334	<0.001	Yes
0 vs. 0.0156	0.406	0.271	0.790	No

Figure 7.56 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Anacardium occidentale* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.59 ± 0.03 µg/mL. Concentration of *A. occidentale* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

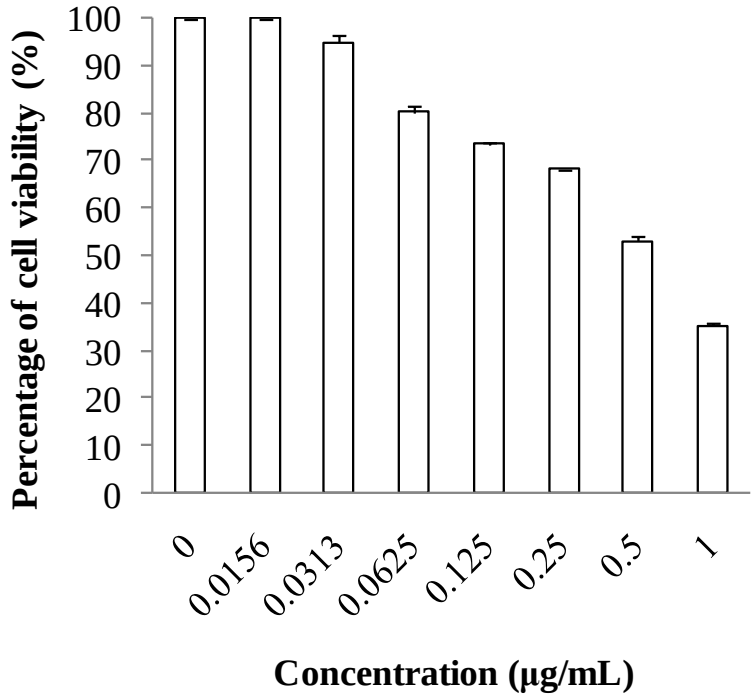


Figure 7.56 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *A. occidentale* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Anacardium occidentale* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.407)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	35.243	0.427	0.247
0.5	3	0	53.101	1.095	0.632
0.25	3	0	68.111	0.164	0.0947
0.125	3	0	73.567	0.268	0.155
0.0625	3	0	80.321	1.270	0.733
0.0313	3	0	95.082	1.679	0.969
0.0156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	11362.946	1623.278	2196.866	<0.001
Residual	16	11.822	0.739		
Total	23	11374.769			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	64.757	92.266	<0.001	Yes
0 vs. 0.5	46.899	66.821	<0.001	Yes
0 vs. 0.25	31.889	45.435	<0.001	Yes
0 vs. 0.125	26.433	37.661	<0.001	Yes
0 vs. 0.0625	19.679	28.038	<0.001	Yes
0 vs. 0.0313	4.918	7.007	<0.001	Yes
0 vs. 0.0156	0.000	0.000	1.000	No

Figure 7.57 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Andrographis paniculata* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.25 ± 0.01 µg/mL. Concentration of *A. paniculata* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

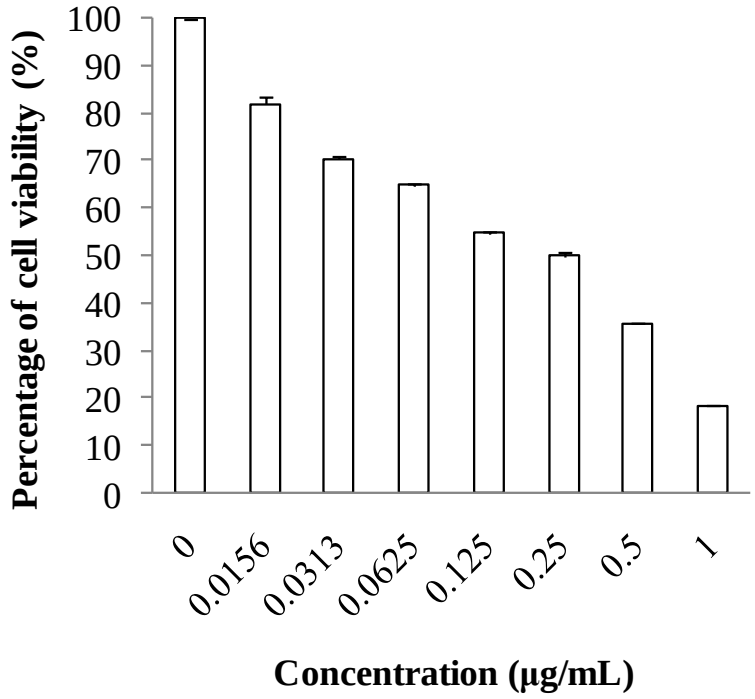


Figure 7.57 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *A. paniculata* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Andrographis paniculata* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.257)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	18.255	0.209	0.121
0.5	3	0	35.592	0.293	0.169
0.25	3	0	49.932	0.621	0.359
0.125	3	0	54.770	0.421	0.243
0.0625	3	0	64.926	0.255	0.147
0.0313	3	0	70.442	0.621	0.359
0.0156	3	0	81.927	1.652	0.954
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	14037.184	2005.312	4141.143	<0.001
Residual	16	7.748	0.484		
Total	23	14044.932			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	81.745	143.872	<0.001	Yes
0 vs. 0.5	64.408	113.359	<0.001	Yes
0 vs. 0.25	50.068	88.120	<0.001	Yes
0 vs. 0.125	45.230	79.605	<0.001	Yes
0 vs. 0.0625	35.074	61.731	<0.001	Yes
0 vs. 0.0313	29.558	52.022	<0.001	Yes
0 vs. 0.0156	18.073	31.809	<0.001	Yes

Figure 7.58 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Cinnamomum zeylanicum* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.22 ± 0.00 µg/mL. Concentration of *C. zeylanicum* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

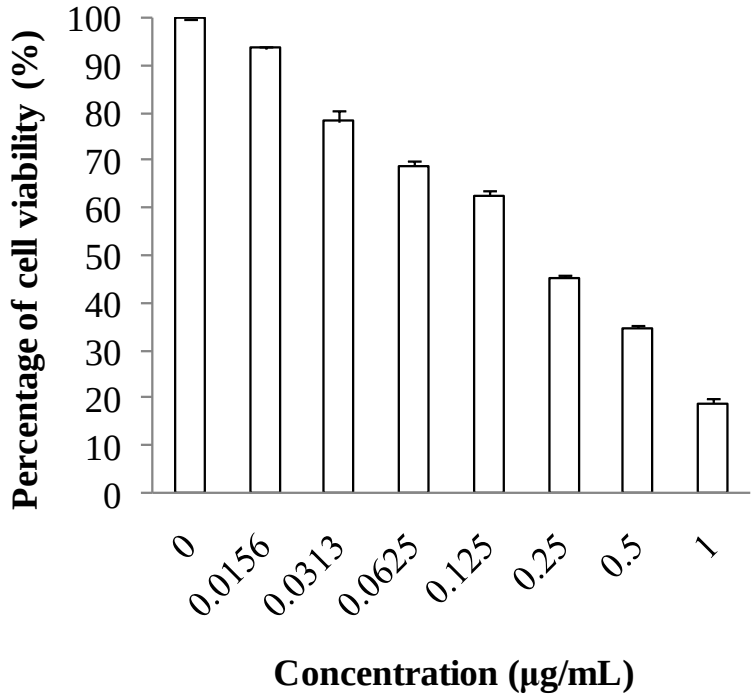


Figure 7.58 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *C. zeylanicum* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Cinnamomum zeylanicum* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.562)

Equal Variance Test: Passed (P = 0.287)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	18.812	1.214	0.701
0.5	3	0	34.639	0.610	0.352
0.25	3	0	45.244	0.560	0.324
0.125	3	0	62.669	0.857	0.495
0.0625	3	0	68.918	1.087	0.628
0.0313	3	0	78.454	2.119	1.223
0.0156	3	0	93.839	0.189	0.109
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	16997.902	2428.272	2259.206	<0.001
Residual	16	17.197	1.075		
Total	23	17015.099			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	81.188	95.911	<0.001	Yes
0 vs. 0.5	65.361	77.213	<0.001	Yes
0 vs. 0.25	54.756	64.685	<0.001	Yes
0 vs. 0.125	37.331	44.101	<0.001	Yes
0 vs. 0.0625	31.082	36.718	<0.001	Yes
0 vs. 0.0313	21.546	25.453	<0.001	Yes
0 vs. 0.0156	6.161	7.278	<0.001	Yes

Figure 7.59 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Euodia redlevi* (fruit&flower) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.41 ± 0.01 µg/mL. Concentration of *E. redleri* (fruit&flower) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

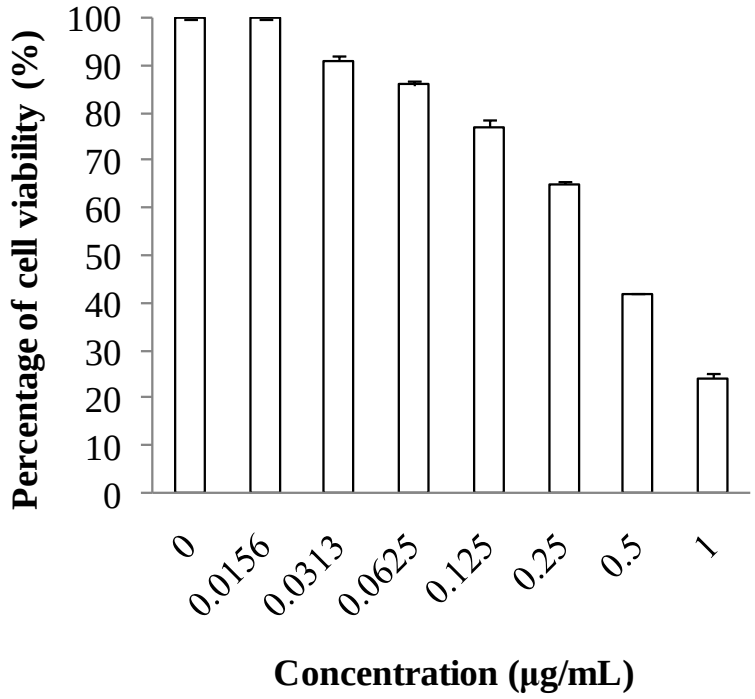


Figure 7.59 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *E. redleri* (fruit&flower) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Euodia redlevi* (fruit&flower) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.542)

Equal Variance Test: Passed (P = 0.636)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	24.028	1.274	0.735
0.5	3	0	41.885	0.386	0.223
0.25	3	0	65.156	0.637	0.368
0.125	3	0	77.142	1.533	0.885
0.0625	3	0	86.103	0.761	0.439
0.0313	3	0	91.097	1.325	0.765
0.0156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	16206.241	2315.177	2699.412	<0.001
Residual	16	13.723	0.858		
Total	23	16219.963			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	75.972	100.471	<0.001	Yes
0 vs. 0.5	58.115	76.856	<0.001	Yes
0 vs. 0.25	34.844	46.080	<0.001	Yes
0 vs. 0.125	22.858	30.229	<0.001	Yes
0 vs. 0.0625	13.897	18.378	<0.001	Yes
0 vs. 0.0313	8.903	11.773	<0.001	Yes
0 vs. 0.0156	0.000	0.000	1.000	No

Figure 7.60 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Garcinia atroviridis* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.33 ± 0.02 µg/mL. Concentration of *G. atroviridis* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

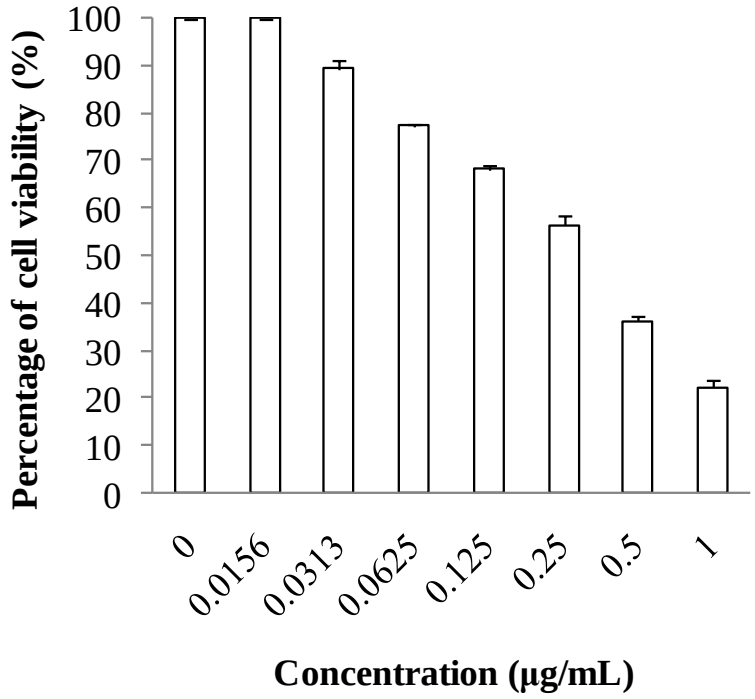


Figure 7.60 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *G. atroviridis* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia atroviridis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.274)

Equal Variance Test: Passed (P = 0.647)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	22.157	1.361	0.786
0.5	3	0	36.216	0.911	0.526
0.25	3	0	56.401	1.848	1.067
0.125	3	0	68.328	0.767	0.443
0.0625	3	0	77.302	0.661	0.382
0.0313	3	0	89.517	1.875	1.082
0.0156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	17518.578	2502.654	1882.118	<0.001
Residual	16	21.275	1.330		
Total	23	17539.853			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	77.843	82.677	<0.001	Yes
0 vs. 0.5	63.784	67.746	<0.001	Yes
0 vs. 0.25	43.599	46.306	<0.001	Yes
0 vs. 0.125	31.672	33.639	<0.001	Yes
0 vs. 0.0625	22.698	24.108	<0.001	Yes
0 vs. 0.0313	10.483	11.135	<0.001	Yes
0 vs. 0.0156	0.000	0.000	1.000	No

Figure 7.61 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Garcinia mangostana* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.39 ± 0.01 µg/mL. Concentration of *G. mangostana* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

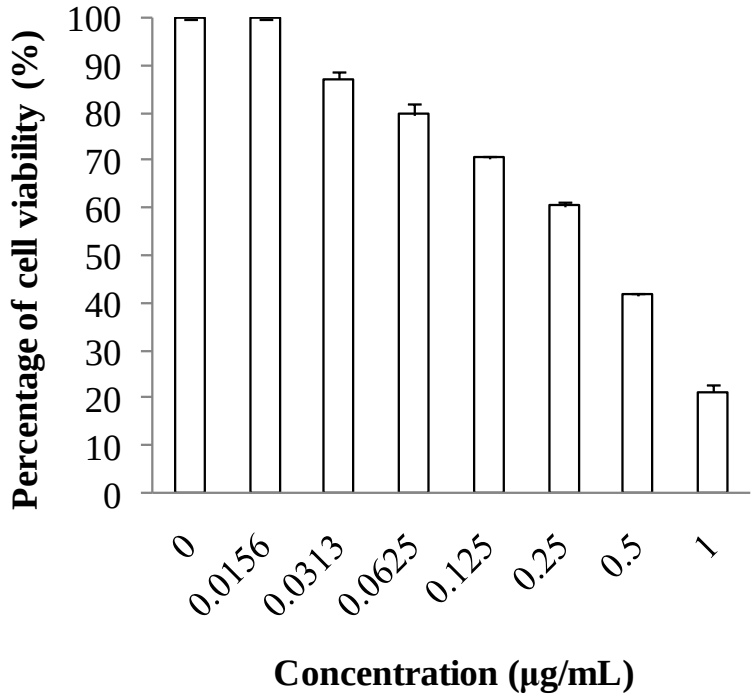


Figure 7.61 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *G. mangostana* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.056)

Equal Variance Test: Passed (P = 0.144)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	21.312	1.566	0.904
0.5	3	0	41.772	0.400	0.231
0.25	3	0	60.587	0.556	0.321
0.125	3	0	70.610	0.448	0.259
0.0625	3	0	79.872	2.426	1.401
0.0313	3	0	87.256	1.636	0.945
0.0156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	16354.072	2336.296	1599.407	<0.001
Residual	16	23.372	1.461		
Total	23	16377.443			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	78.688	79.739	<0.001	Yes
0 vs. 0.5	58.228	59.006	<0.001	Yes
0 vs. 0.25	39.413	39.939	<0.001	Yes
0 vs. 0.125	29.390	29.783	<0.001	Yes
0 vs. 0.0625	20.128	20.397	<0.001	Yes
0 vs. 0.0313	12.744	12.914	<0.001	Yes
0 vs. 0.0156	0.000	0.000	1.000	No

Figure 7.62 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Garcinia mangostana* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.30 ± 0.01 µg/mL. Concentration of *G. mangostana* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

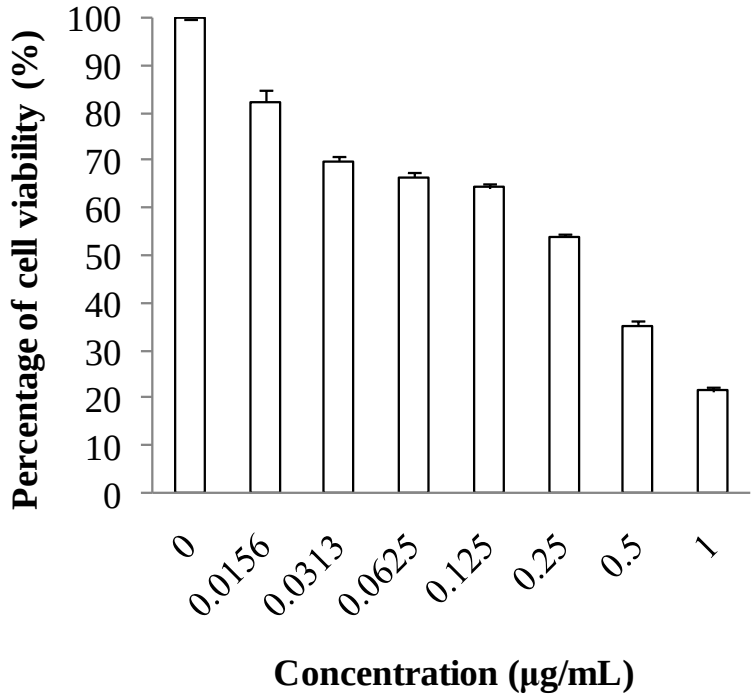


Figure 7.62 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *G. mangostana* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.918)

Equal Variance Test: Passed (P = 0.565)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	21.337	0.839	0.485
0.5	3	0	35.142	1.220	0.704
0.25	3	0	53.880	0.895	0.517
0.125	3	0	64.421	0.866	0.500
0.0625	3	0	66.521	0.941	0.543
0.0313	3	0	69.957	1.251	0.722
0.0156	3	0	82.412	2.400	1.386
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	13171.163	1881.595	1259.036	<0.001
Residual	16	23.912	1.494		
Total	23	13195.075			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	78.663	78.809	<0.001	Yes
0 vs. 0.5	64.858	64.978	<0.001	Yes
0 vs. 0.25	46.120	46.205	<0.001	Yes
0 vs. 0.125	35.579	35.645	<0.001	Yes
0 vs. 0.0625	33.479	33.541	<0.001	Yes
0 vs. 0.0313	30.043	30.098	<0.001	Yes
0 vs. 0.0156	17.588	17.621	<0.001	Yes

Figure 7.63 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Hibiscus tiliaceus* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.33 ± 0.01 µg/mL. Concentration of *H. tiliaceus* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

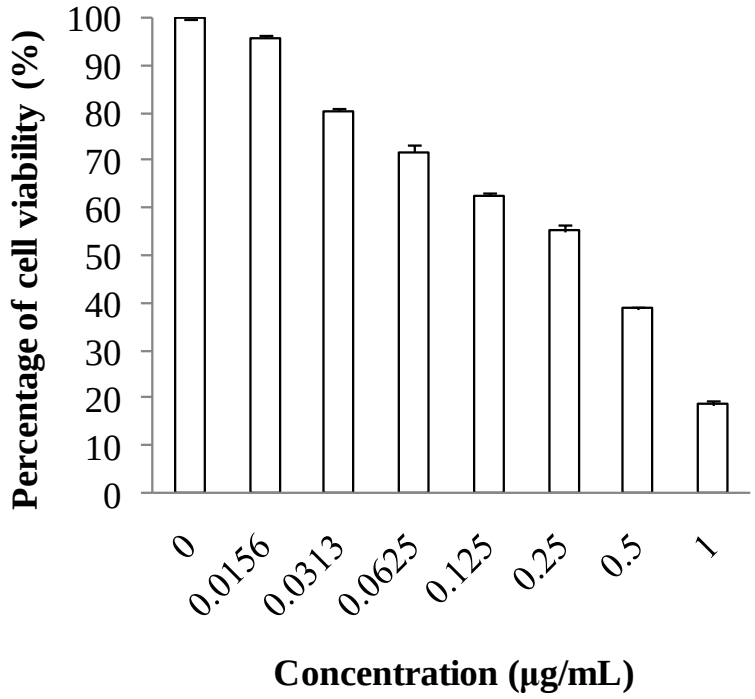


Figure 7.63 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *H. tiliaceus* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Hibiscus tiliaceus* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.220)

Equal Variance Test: Passed (P = 0.821)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	18.571	0.661	0.382
0.5	3	0	38.741	0.231	0.133
0.25	3	0	55.268	1.122	0.648
0.125	3	0	62.748	0.708	0.409
0.0625	3	0	71.748	1.634	0.944
0.0313	3	0	80.539	0.643	0.371
0.0156	3	0	95.849	0.893	0.515
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	16221.222	2317.317	3023.233	<0.001
Residual	16	12.264	0.767		
Total	23	16233.486			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	81.429	113.911	<0.001	Yes
0 vs. 0.5	61.259	85.696	<0.001	Yes
0 vs. 0.25	44.732	62.576	<0.001	Yes
0 vs. 0.125	37.252	52.112	<0.001	Yes
0 vs. 0.0625	28.252	39.522	<0.001	Yes
0 vs. 0.0313	19.461	27.225	<0.001	Yes
0 vs. 0.0156	4.151	5.807	<0.001	Yes

Figure 7.64 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Lawsonia inermis* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.26 ± 0.02 µg/mL. Concentration of *L. inermis* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

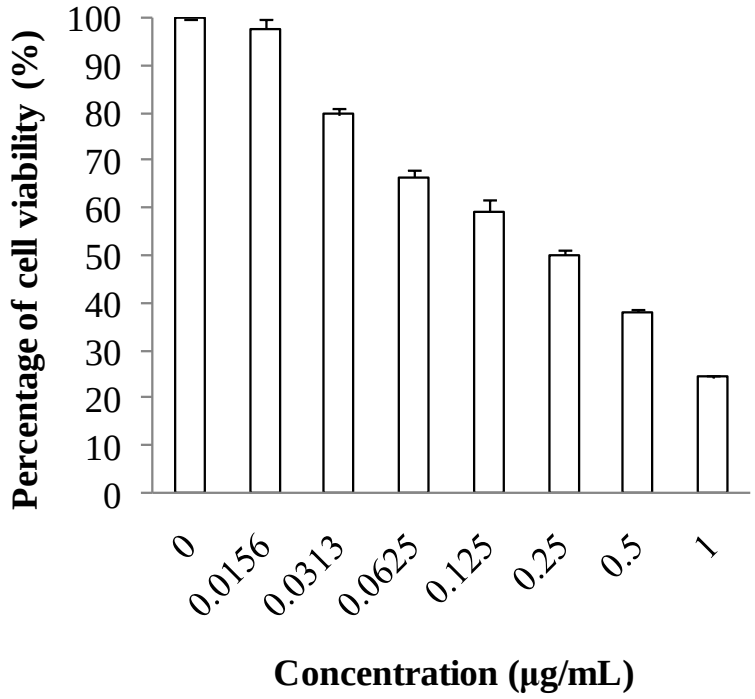


Figure 7.64 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *L. inermis* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Lawsonia inermi*s (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.579)

Equal Variance Test: Passed (P = 0.654)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	24.328	0.401	0.232
0.5	3	0	38.081	0.826	0.477
0.25	3	0	50.256	0.873	0.504
0.125	3	0	59.346	2.280	1.317
0.0625	3	0	66.549	1.614	0.932
0.0313	3	0	79.850	1.494	0.863
0.0156	3	0	97.927	1.978	1.142
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	15475.849	2210.836	1137.063	<0.001
Residual	16	31.109	1.944		
Total	23	15506.959			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	75.672	66.465	<0.001	Yes
0 vs. 0.5	61.919	54.386	<0.001	Yes
0 vs. 0.25	49.744	43.692	<0.001	Yes
0 vs. 0.125	40.654	35.708	<0.001	Yes
0 vs. 0.0625	33.451	29.381	<0.001	Yes
0 vs. 0.0313	20.150	17.699	<0.001	Yes
0 vs. 0.0156	2.073	1.821	0.087	No

Figure 7.65 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Leptospermum flavescens* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.39 ± 0.01 µg/mL. Concentration of *L. flavescens* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

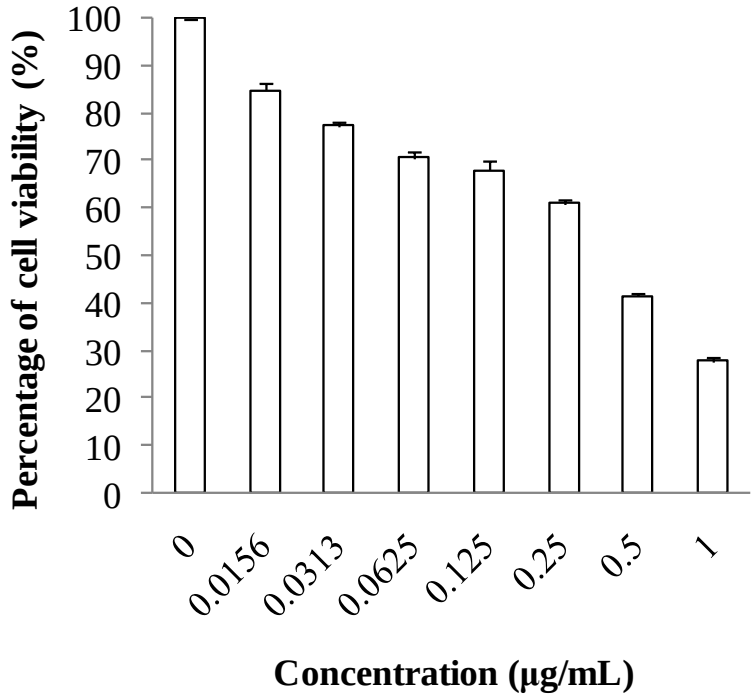


Figure 7.65 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *L. flavescens* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Leptospermum flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.351)

Equal Variance Test: Passed (P = 0.618)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	27.825	0.547	0.316
0.5	3	0	41.363	0.522	0.302
0.25	3	0	61.010	0.833	0.481
0.125	3	0	67.942	2.126	1.228
0.0625	3	0	70.596	1.269	0.733
0.0313	3	0	77.469	0.752	0.434
0.0156	3	0	84.835	1.625	0.938
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	11265.828	1609.404	1214.139	<0.001
Residual	16	21.209	1.326		
Total	23	11287.036			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	72.175	76.778	<0.001	Yes
0 vs. 0.5	58.637	62.377	<0.001	Yes
0 vs. 0.25	38.990	41.477	<0.001	Yes
0 vs. 0.125	32.058	34.102	<0.001	Yes
0 vs. 0.0625	29.404	31.279	<0.001	Yes
0 vs. 0.0313	22.531	23.968	<0.001	Yes
0 vs. 0.0156	15.165	16.132	<0.001	Yes

Figure 7.66 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Oxalis barrelieri* (root) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.33 ± 0.01 µg/mL. Concentration of *O. barrelieri* (root) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

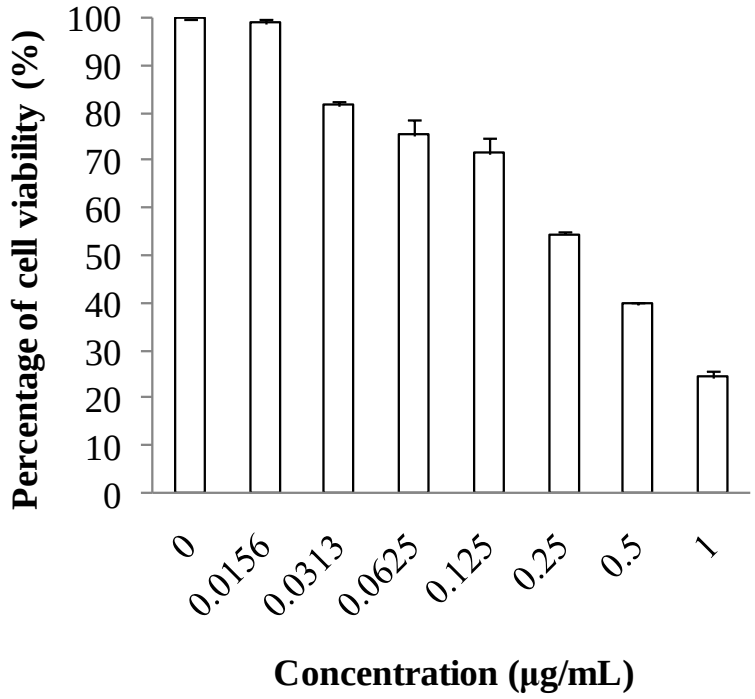


Figure 7.66 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *O. barrelieri* (root) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Oxalis barrelieri* (root) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.127)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	24.263	1.392	0.804
0.5	3	0	39.719	0.233	0.135
0.25	3	0	54.550	0.595	0.343
0.125	3	0	71.617	3.237	1.869
0.0625	3	0	75.394	3.197	1.846
0.0313	3	0	81.766	0.840	0.485
0.0156	3	0	99.178	0.855	0.494
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	15438.622	2205.517	720.784	<0.001
Residual	16	48.958	3.060		
Total	23	15487.581			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	75.737	53.027	<0.001	Yes
0 vs. 0.5	60.281	42.206	<0.001	Yes
0 vs. 0.25	45.450	31.822	<0.001	Yes
0 vs. 0.125	28.383	19.873	<0.001	Yes
0 vs. 0.0625	24.606	17.228	<0.001	Yes
0 vs. 0.0313	18.234	12.767	<0.001	Yes
0 vs. 0.0156	0.822	0.576	0.573	No

Figure 7.67 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Peltophorum pterocarpum* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.48 ± 0.02 µg/mL. Concentration of *P. pterocarpum* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

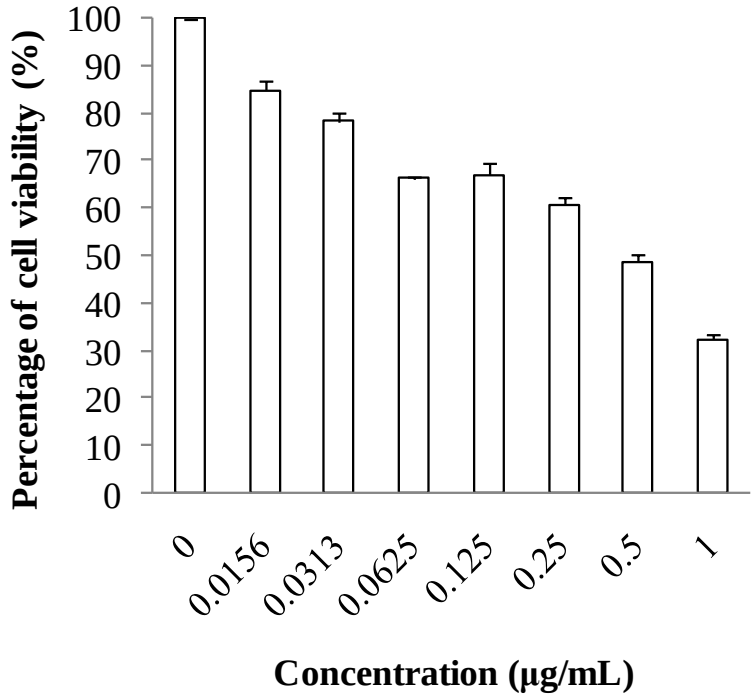


Figure 7.67 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *P. pterocarpum* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Peltophorum pterocarpum* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.536)

Equal Variance Test: Passed (P = 0.382)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	32.302	1.294	0.747
0.5	3	0	48.683	1.478	0.854
0.25	3	0	60.742	1.532	0.884
0.125	3	0	67.020	2.519	1.455
0.0625	3	0	66.260	0.549	0.317
0.0313	3	0	78.283	1.748	1.009
0.0156	3	0	84.975	2.173	1.255
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	9353.775	1336.254	518.038	<0.001
Residual	16	41.271	2.579		
Total	23	9395.047			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	67.698	51.624	<0.001	Yes
0 vs. 0.5	51.317	39.133	<0.001	Yes
0 vs. 0.25	39.258	29.937	<0.001	Yes
0 vs. 0.0625	33.740	25.729	<0.001	Yes
0 vs. 0.125	32.980	25.150	<0.001	Yes
0 vs. 0.0313	21.717	16.561	<0.001	Yes
0 vs. 0.0156	15.025	11.458	<0.001	Yes

Figure 7.68 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with *Tetracera indica* (fruit) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.30 ± 0.04 µg/mL. Concentration of *T. indica* (fruit) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

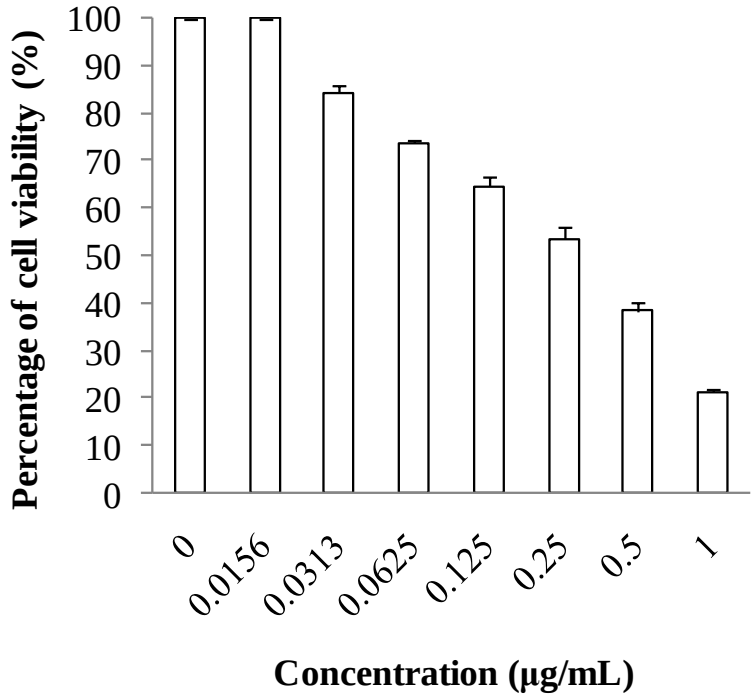


Figure 7.68 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with *T. indica* (fruit) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Tetracera indica* (fruit) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.214)

Equal Variance Test: Passed (P = 0.942)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	21.215	0.592	0.342
0.5	3	0	38.306	1.641	0.947
0.25	3	0	53.425	2.675	1.545
0.125	3	0	64.643	2.060	1.189
0.0625	3	0	73.677	0.849	0.490
0.0313	3	0	84.483	1.419	0.819
0.0156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	16913.084	2416.155	1125.371	<0.001
Residual	16	34.352	2.147		
Total	23	16947.436			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	78.785	65.853	<0.001	Yes
0 vs. 0.5	61.694	51.567	<0.001	Yes
0 vs. 0.25	46.575	38.930	<0.001	Yes
0 vs. 0.125	35.357	29.553	<0.001	Yes
0 vs. 0.0625	26.323	22.002	<0.001	Yes
0 vs. 0.0313	15.517	12.970	<0.001	Yes
0 vs. 0.0156	0.000	0.000	1.000	No

Figure 7.69 shows *in vitro* cytotoxicity effects of doxorubicin hydrochloride, in combination with 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of doxorubicin hydrochloride generated from the dose-response graph is 0.26 ± 0.00 µg/mL. Concentration of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of doxorubicin hydrochloride varied (0-1 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

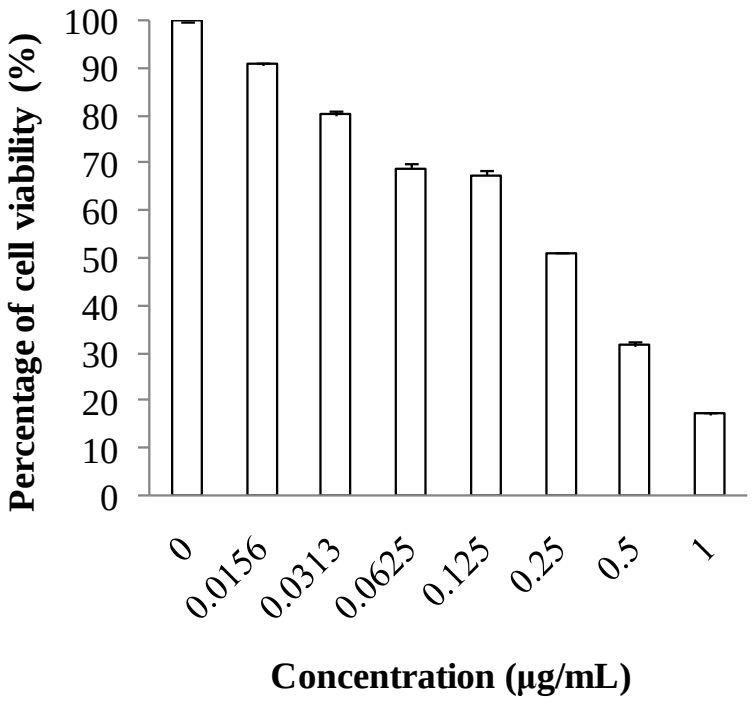


Figure 7.69 : Cytotoxicity effects of doxorubicin hydrochloride, in combination with 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.205)

Equal Variance Test: Passed (P = 0.559)

Group Name	N	Missing	Mean	Std Dev	SEM
1	3	0	17.183	0.195	0.112
0.5	3	0	31.531	0.822	0.474
0.25	3	0	51.043	0.225	0.130
0.125	3	0	67.409	0.974	0.562
0.0625	3	0	68.986	1.040	0.600
0.0313	3	0	80.349	0.552	0.319
0.0156	3	0	90.871	0.393	0.227
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	17199.237	2457.034	6041.853	<0.001
Residual	16	6.507	0.407		
Total	23	17205.743			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 1	82.817	159.055	<0.001	Yes
0 vs. 0.5	68.469	131.497	<0.001	Yes
0 vs. 0.25	48.957	94.024	<0.001	Yes
0 vs. 0.125	32.591	62.592	<0.001	Yes
0 vs. 0.0625	31.014	59.564	<0.001	Yes
0 vs. 0.0313	19.651	37.741	<0.001	Yes
0 vs. 0.0156	9.129	17.533	<0.001	Yes

One Way Analysis of Variance

Data source: Overall IC50 for doxo combination

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.363)

Group Name	N	Missing	Mean	Std Dev	SEM
Doxo alone	3	0	0.788	0.0459	0.0265
Doxo + Ethacrynic acid	3	0	0.187	0.0108	0.00623
Doxo + Anacardium occidentale	3	0	0.586	0.0249	0.0144
Doxo + Andrographis paniculata	3	0	0.246	0.0123	0.00713
Doxo + Cinnamomum zeylanicu	3	0	0.216	0.00208	0.00120
Doxo + Euodia redlevi (fruit&f	3	0	0.413	0.00529	0.00306
Doxo + Garcinia atroviridis (b	3	0	0.328	0.0189	0.0109
Doxo + Garcinia mangostana (br	3	0	0.391	0.00643	0.00371
Doxo + Garcinia mangostana (le	3	0	0.302	0.0110	0.00633
Doxo + Hibiscus tiliaceus (lea	3	0	0.329	0.0131	0.00755
Doxo + Lawsonia inermis (branc	3	0	0.256	0.0159	0.00917
Doxo + Leptospermum flavesce	3	0	0.390	0.00819	0.00473
Doxo + Oxalis barrelieri (root	3	0	0.327	0.00808	0.00467
Doxo + Peltophorum pterocarpu	3	0	0.476	0.0227	0.0131
Doxo + Tetracera indica (fruit	3	0	0.304	0.0420	0.0242
Doxo + 50% MeOH 2% acetic a	3	0	0.263	0.00208	0.00120

Source of Variation	DF	SS	MS	F	P
Between Groups	15	1.037	0.0692	173.176	<0.001
Residual	32	0.0128	0.000399		
Total	47	1.050			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Doxo alone vs. Doxo + Ethac	0.601	36.833	<0.001	Yes
Doxo alone vs. Doxo + Cinna	0.573	35.097	<0.001	Yes
Doxo alone vs. Doxo + Andro	0.542	33.217	<0.001	Yes
Doxo alone vs. Doxo + Lawso	0.532	32.605	<0.001	Yes
Doxo alone vs. Doxo + 50% M	0.526	32.216	<0.001	Yes
Doxo alone vs. Doxo + Garci	0.487	29.826	<0.001	Yes
Doxo alone vs. Doxo + Tetra	0.484	29.683	<0.001	Yes
Doxo alone vs. Doxo + Oxali	0.462	28.294	<0.001	Yes
Doxo alone vs. Doxo + Garci	0.460	28.192	<0.001	Yes
Doxo alone vs. Doxo + Hibis	0.459	28.151	<0.001	Yes

Doxo alone vs. Doxo + Lepto	0.398	24.413	<0.001	Yes
Doxo alone vs. Doxo + Garci	0.398	24.372	<0.001	Yes
Doxo alone vs. Doxo + Euodi	0.375	23.003	<0.001	Yes
Doxo alone vs. Doxo + Pelto	0.312	19.142	<0.001	Yes
Doxo alone vs. Doxo + Anaca	0.203	12.421	<0.001	Yes

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
Doxo + Ethac vs. Doxo + Anaca	0.398	28.884	<0.001	Yes
Doxo + Ethac vs. Doxo + Pelto	0.289	20.931	<0.001	Yes
Doxo + Ethac vs. Doxo + Euodi	0.226	16.363	<0.001	Yes
Doxo + Ethac vs. Doxo + Garci	0.203	14.744	<0.001	Yes
Doxo + Ethac vs. Doxo + Lepto	0.203	14.696	<0.001	Yes
Doxo + Ethac vs. Doxo + Hibis	0.142	10.272	<0.001	Yes
Doxo + Ethac vs. Doxo + Garci	0.141	10.224	<0.001	Yes
Doxo + Ethac vs. Doxo + Oxali	0.139	10.103	<0.001	Yes
Doxo + Ethac vs. Doxo + Tetra	0.117	8.460	<0.001	Yes
Doxo + Ethac vs. Doxo + Garci	0.114	8.290	<0.001	Yes
Doxo + Ethac vs. Doxo + 50% M	0.0753	5.462	<0.001	Yes
Doxo + Ethac vs. Doxo + Lawso	0.0690	5.003	<0.001	Yes
Doxo + Ethac vs. Doxo + Andro	0.0590	4.278	<0.001	Yes
Doxo + Ethac vs. Doxo + Cinna	0.0283	2.054	0.049	Yes

COMBINATION EFFECTS OF PLANT SAMPLES AND CISPLATIN ON CYTOTOXICITY OF HT-29 CELLS

Figure 7.70 shows *in vitro* cytotoxicity effects of cisplatin, in combination with ethacrynic acid, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.78 ± 0.15 μg/mL. Concentration of ethacrynic acid was fixed at 10 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

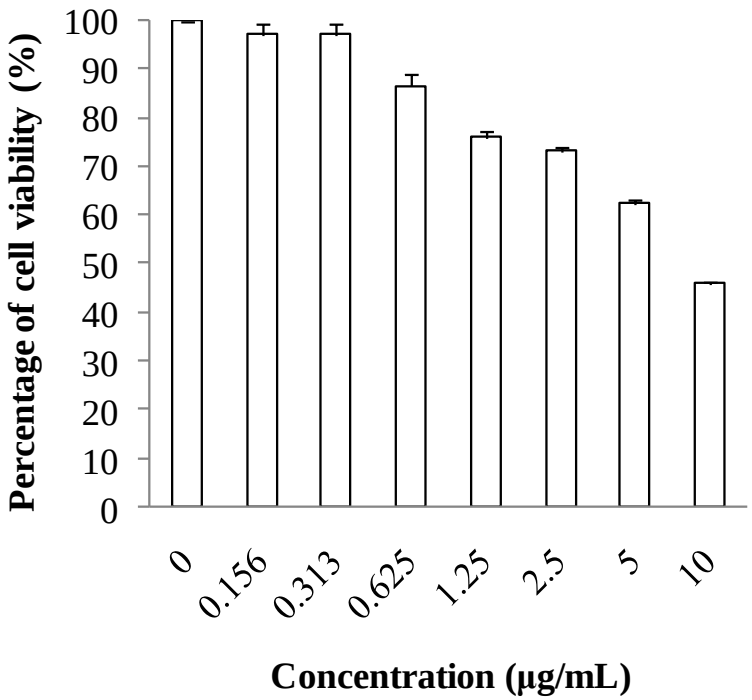


Figure 7.70 : Cytotoxicity effects of cisplatin, in combination with ethacrynic acid, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: Ethacrynic acid

Normality Test (Shapiro-Wilk) Passed (P = 0.331)

Equal Variance Test: Passed (P = 0.590)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	45.999	0.508	0.293
5	3	0	62.391	0.736	0.425
2.5	3	0	73.206	0.679	0.392
1.25	3	0	76.061	1.339	0.773
.626	3	0	86.765	2.501	1.444
.313	3	0	97.231	2.166	1.250
.156	3	0	97.346	2.023	1.168
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	7713.697	1101.957	487.296	<0.001
Residual	16	36.182	2.261		
Total	23	7749.878			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	54.001	43.981	<0.001	Yes
0 vs. 5	37.609	30.630	<0.001	Yes
0 vs. 2.5	26.794	21.822	<0.001	Yes
0 vs. 1.25	23.939	19.497	<0.001	Yes
0 vs. .626	13.235	10.779	<0.001	Yes
0 vs. .313	2.769	2.256	0.075	No
0 vs. .156	2.654	2.161	0.046	Yes

Figure 7.71 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Anacardium occidentale* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.66 ± 0.13 µg/mL. Concentration of *A. occidentale* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of cisplatin varied (0-10 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

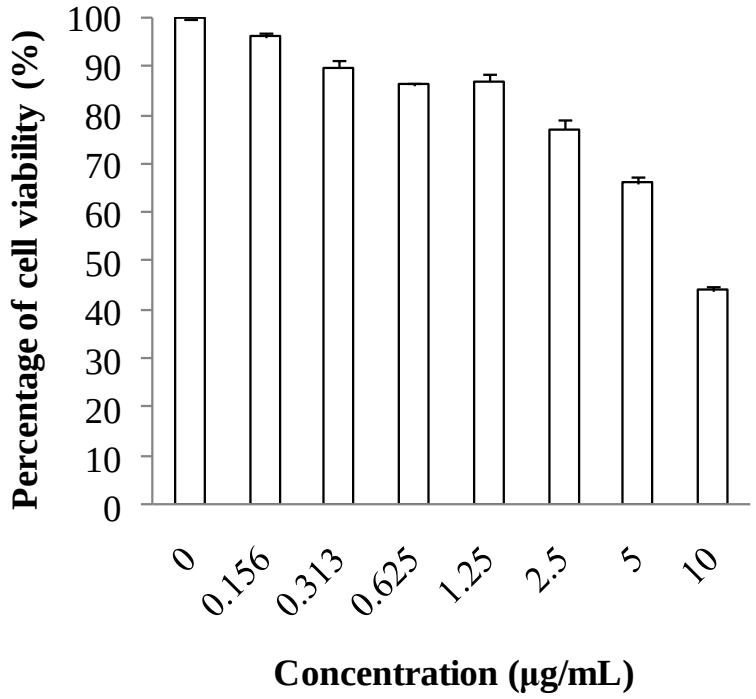


Figure 7.71 : Cytotoxicity effects of cisplatin, in combination with *A. occidentale* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Anacardium occidentale* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.656)

Equal Variance Test: Passed (P = 0.935)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	44.076	0.900	0.520
5	3	0	66.153	1.204	0.695
2.5	3	0	77.359	1.708	0.986
1.25	3	0	87.205	1.279	0.739
.626	3	0	86.414	0.573	0.331
.313	3	0	89.972	1.421	0.820
.156	3	0	96.287	0.977	0.564
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	7019.977	1002.854	793.294	<0.001
Residual	16	20.227	1.264		
Total	23	7040.203			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	55.924	60.917	<0.001	Yes
0 vs. 5	33.847	36.869	<0.001	Yes
0 vs. 2.5	22.641	24.662	<0.001	Yes
0 vs. .626	13.586	14.799	<0.001	Yes
0 vs. 1.25	12.795	13.938	<0.001	Yes
0 vs. .313	10.028	10.923	<0.001	Yes
0 vs. .156	3.713	4.045	<0.001	Yes

Figure 7.72 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Andrographis paniculata* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 4.70 ± 0.18 μg/mL. Concentration of *A. paniculata* (leaf) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

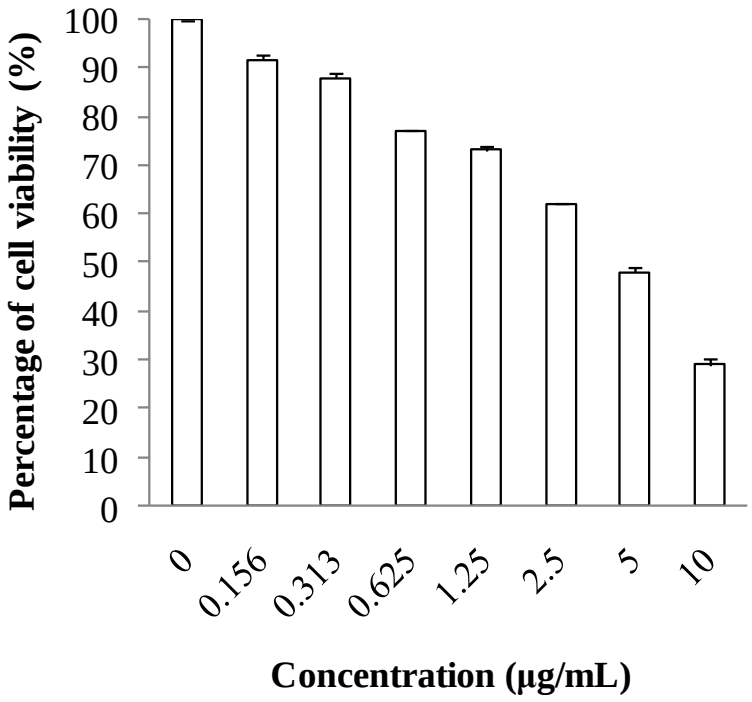


Figure 7.72 : Cytotoxicity effects of cisplatin, in combination with *A. paniculata* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Andrographis paniculata* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.097)

Equal Variance Test: Passed (P = 0.280)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	29.058	1.171	0.676
5	3	0	48.248	1.122	0.648
2.5	3	0	62.325	0.0915	0.0528
1.25	3	0	73.391	0.809	0.467
.626	3	0	77.245	0.171	0.0987
.313	3	0	88.169	1.073	0.620
.156	3	0	91.747	1.206	0.696
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	11887.225	1698.175	2291.403	<0.001
Residual	16	11.858	0.741		
Total	23	11899.083			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	70.942	100.928	<0.001	Yes
0 vs. 5	51.752	73.626	<0.001	Yes
0 vs. 2.5	37.675	53.599	<0.001	Yes
0 vs. 1.25	26.609	37.856	<0.001	Yes
0 vs. .626	22.755	32.374	<0.001	Yes
0 vs. .313	11.831	16.832	<0.001	Yes
0 vs. .156	8.253	11.741	<0.001	Yes

Figure 7.73 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Cinnamomum zeylanicum* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 9.23 ± 0.07 μg/mL. Concentration of *C. zeylanicum* (branch) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

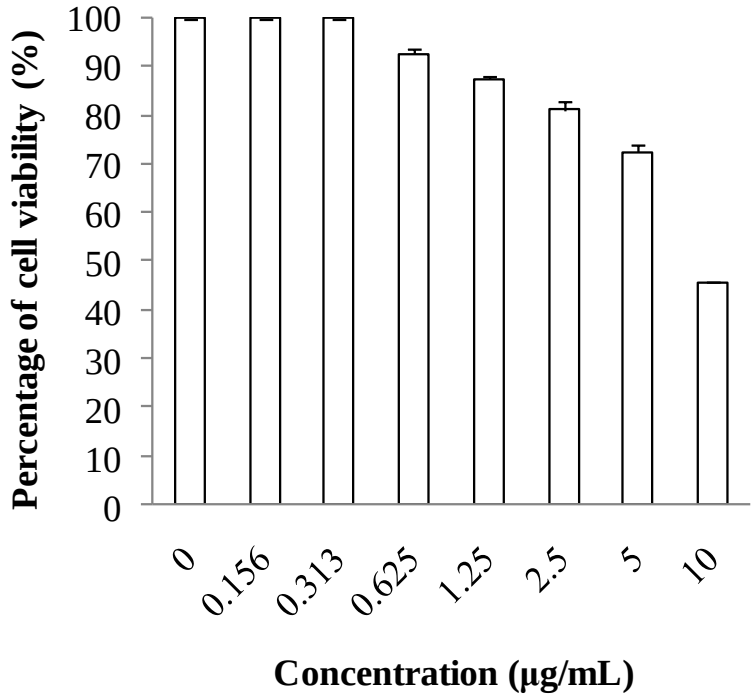


Figure 7.73 : Cytotoxicity effects of cisplatin, in combination with *C. zeylanicum* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Cinnamomum zeylanicum* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.701)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	45.939	0.209	0.121
5	3	0	72.503	1.590	0.918
2.5	3	0	81.272	1.844	1.065
1.25	3	0	87.604	0.810	0.468
.626	3	0	92.709	0.908	0.524
.313	3	0	100.000	0.000	0.000
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	7311.100	1044.443	1121.029	<0.001
Residual	16	14.907	0.932		
Total	23	7326.007			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	54.061	68.595	<0.001	Yes
0 vs. 5	27.497	34.890	<0.001	Yes
0 vs. 2.5	18.728	23.764	<0.001	Yes
0 vs. 1.25	12.396	15.728	<0.001	Yes
0 vs. .626	7.291	9.251	<0.001	Yes
0 vs. .313	0.000	0.000	1.000	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.74 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Euodia redlevi* (fruit&flower) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.55 ± 0.06 μg/mL. Concentration of *E. redleri* (fruit&flower) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

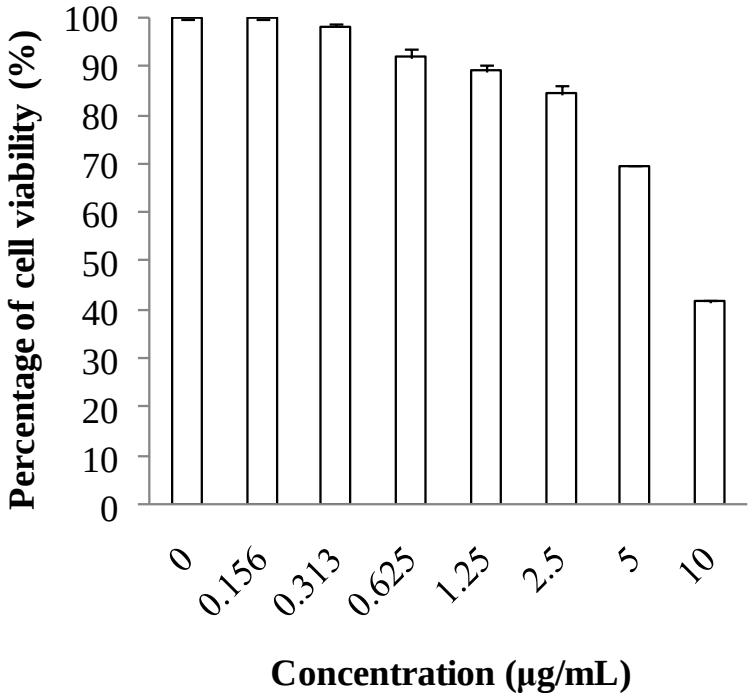


Figure 7.74 : Cytotoxicity effects of cisplatin, in combination with *E. redleri* (fruit&flower) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Euodia redlevi* (fruit&flower) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.325)

Equal Variance Test: Passed (P = 0.640)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	41.924	0.420	0.243
5	3	0	69.704	0.268	0.155
2.5	3	0	84.551	1.669	0.964
1.25	3	0	89.347	1.110	0.641
.626	3	0	92.059	1.628	0.940
.313	3	0	98.458	0.684	0.395
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8362.814	1194.688	1293.814	<0.001
Residual	16	14.774	0.923		
Total	23	8377.588			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	58.076	74.021	<0.001	Yes
0 vs. 5	30.296	38.613	<0.001	Yes
0 vs. 2.5	15.449	19.690	<0.001	Yes
0 vs. 1.25	10.653	13.577	<0.001	Yes
0 vs. .626	7.941	10.121	<0.001	Yes
0 vs. .313	1.542	1.965	0.129	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.75 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Garcinia atroviridis* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.30 ± 0.12 µg/mL. Concentration of *G. atroviridis* (branch) ethanolic extract was fixed at 50 µg/mL while concentration of cisplatin varied (0-10 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

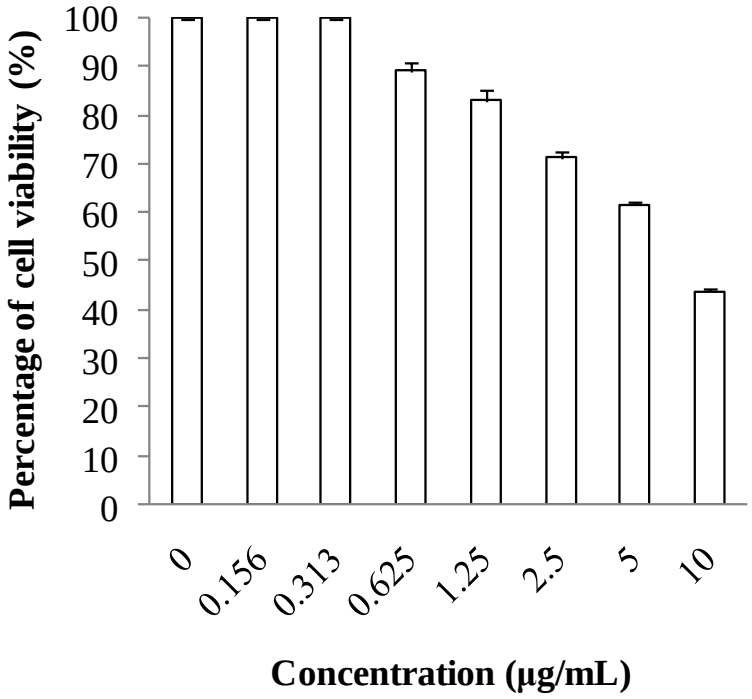


Figure 7.75 : Cytotoxicity effects of cisplatin, in combination with *G. atroviridis* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia atroviridis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.471)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	43.920	0.588	0.339
5	3	0	61.772	0.451	0.260
2.5	3	0	71.421	1.419	0.820
1.25	3	0	83.215	1.937	1.118
.626	3	0	89.231	1.806	1.043
.313	3	0	100.000	0.000	0.000
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8975.235	1282.176	1071.183	<0.001
Residual	16	19.152	1.197		
Total	23	8994.387			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	56.080	62.778	<0.001	Yes
0 vs. 5	38.228	42.794	<0.001	Yes
0 vs. 2.5	28.579	31.993	<0.001	Yes
0 vs. 1.25	16.785	18.790	<0.001	Yes
0 vs. .626	10.769	12.055	<0.001	Yes
0 vs. .313	0.000	0.000	1.000	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.76 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Garcinia mangostana* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 9.24 ± 0.55 μg/mL. Concentration of *G. mangostana* (branch) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

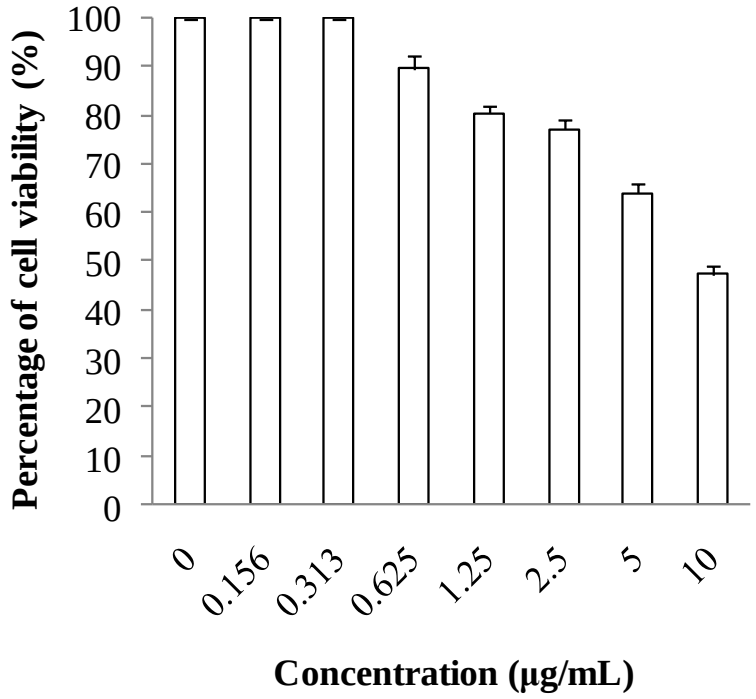


Figure 7.76 : Cytotoxicity effects of cisplatin, in combination with *G. mangostana* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.764)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	47.422	1.898	1.096
5	3	0	64.155	1.773	1.024
2.5	3	0	77.191	2.131	1.231
1.25	3	0	80.617	1.401	0.809
.626	3	0	89.617	2.975	1.718
.313	3	0	100.000	0.000	0.000
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	7704.059	1100.580	398.333	<0.001
Residual	16	44.207	2.763		
Total	23	7748.266			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	52.578	38.740	<0.001	Yes
0 vs. 5	35.845	26.411	<0.001	Yes
0 vs. 2.5	22.809	16.806	<0.001	Yes
0 vs. 1.25	19.383	14.281	<0.001	Yes
0 vs. .626	10.383	7.650	<0.001	Yes
0 vs. .313	0.000	0.000	1.000	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.77 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Garcinia mangostana* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 6.74 ± 0.38 μg/mL. Concentration of *G. mangostana* (leaf) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

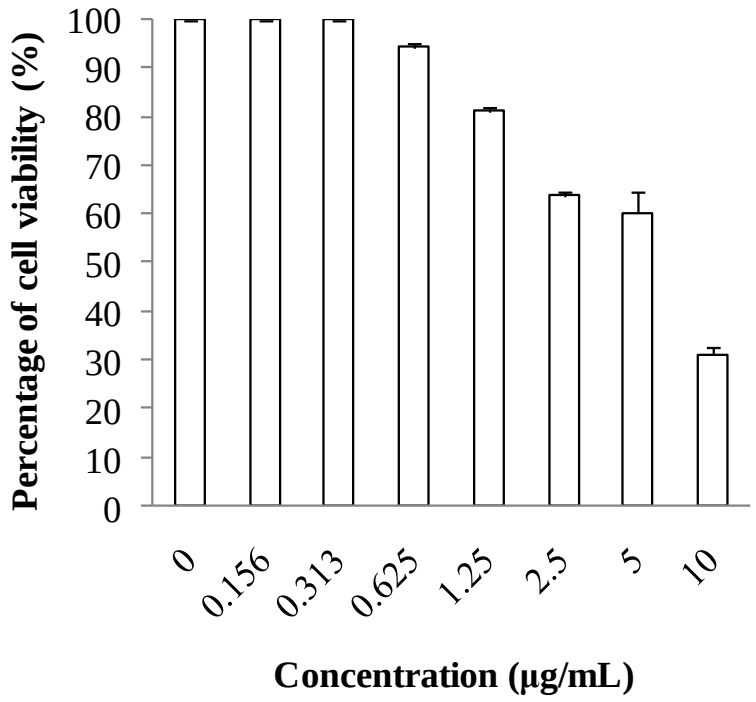


Figure 7.77 : Cytotoxicity effects of cisplatin, in combination with *G. mangostana* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Garcinia mangostana* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.368)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	31.176	1.738	1.003
5	3	0	60.406	4.422	2.553
2.5	3	0	63.952	0.717	0.414
1.25	3	0	81.336	0.662	0.382
.626	3	0	94.412	0.647	0.373
.313	3	0	100.000	0.000	0.000
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	13275.755	1896.536	633.627	<0.001
Residual	16	47.890	2.993		
Total	23	13323.646			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	68.824	48.722	<0.001	Yes
0 vs. 5	39.594	28.030	<0.001	Yes
0 vs. 2.5	36.048	25.519	<0.001	Yes
0 vs. 1.25	18.664	13.213	<0.001	Yes
0 vs. .626	5.588	3.956	0.003	Yes
0 vs. .313	0.000	0.000	1.000	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.78 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Hibiscus tiliaceus* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 9.24 ± 0.30 µg/mL. Concentration of *H. tiliaceus* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of cisplatin varied (0-10 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

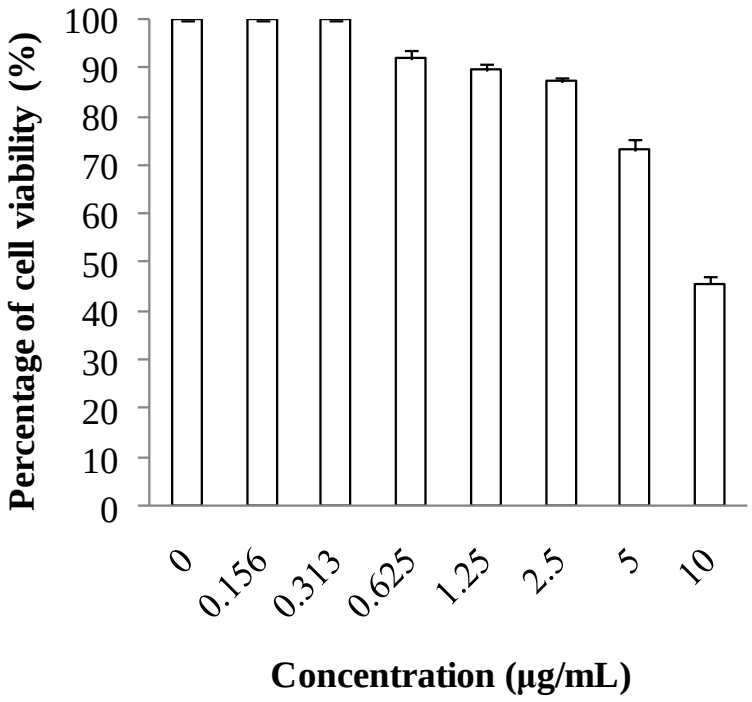


Figure 7.78 : Cytotoxicity effects of cisplatin, in combination with *H. tiliaceus* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Hibiscus tiliaceus* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.165)

Equal Variance Test: Passed (P = 0.817)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	45.809	1.569	0.906
5	3	0	73.218	2.202	1.271
2.5	3	0	87.382	0.882	0.509
1.25	3	0	89.791	1.258	0.726
.626	3	0	92.067	1.569	0.906
.313	3	0	100.000	0.000	0.000
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	7259.192	1037.027	683.955	<0.001
Residual	16	24.260	1.516		
Total	23	7283.451			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	54.191	53.900	<0.001	Yes
0 vs. 5	26.782	26.638	<0.001	Yes
0 vs. 2.5	12.618	12.550	<0.001	Yes
0 vs. 1.25	10.209	10.154	<0.001	Yes
0 vs. .626	7.933	7.890	<0.001	Yes
0 vs. .313	0.000	0.000	1.000	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.79 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Lawsonia inermis* (branch) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 5.46 ± 0.30 μg/mL. Concentration of *L. inermis* (branch) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

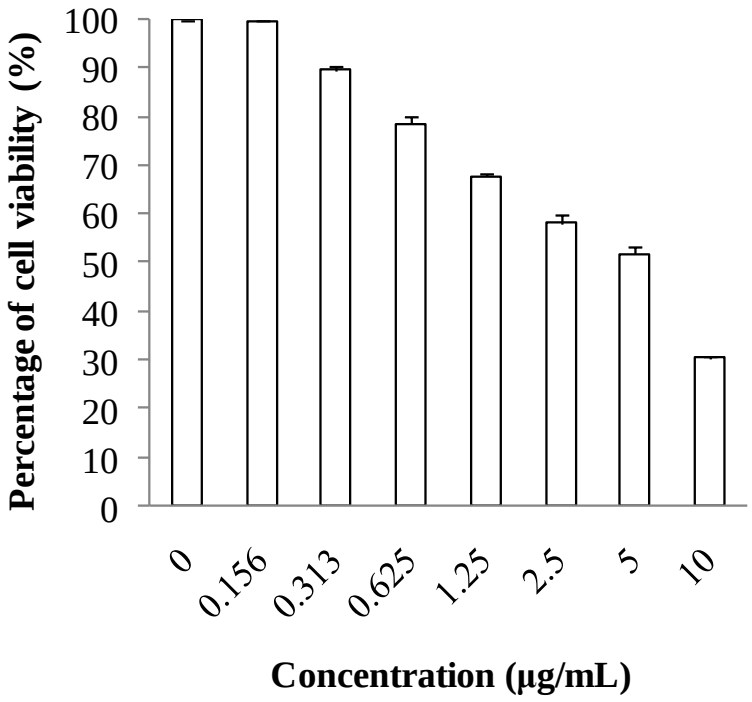


Figure 7.79 : Cytotoxicity effects of cisplatin, in combination with *L. inermis* (branch) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Lawsonia inermis* (branch) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.215)

Equal Variance Test: Passed (P = 0.595)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	30.531	0.534	0.308
5	3	0	51.996	1.413	0.816
2.5	3	0	58.317	1.515	0.875
1.25	3	0	67.871	0.447	0.258
.626	3	0	78.769	1.602	0.925
.313	3	0	89.728	0.667	0.385
.156	3	0	99.703	0.424	0.245
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	12706.778	1815.254	1823.107	<0.001
Residual	16	15.931	0.996		
Total	23	12722.709			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	69.469	85.266	<0.001	Yes
0 vs. 5	48.004	58.920	<0.001	Yes
0 vs. 2.5	41.683	51.161	<0.001	Yes
0 vs. 1.25	32.129	39.435	<0.001	Yes
0 vs. .626	21.231	26.059	<0.001	Yes
0 vs. .313	10.272	12.607	<0.001	Yes
0 vs. .156	0.297	0.365	0.720	No

Figure 7.80 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Leptospermum flavescens* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 6.33 ± 0.40 µg/mL. Concentration of *L. flavescens* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of cisplatin varied (0-10 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

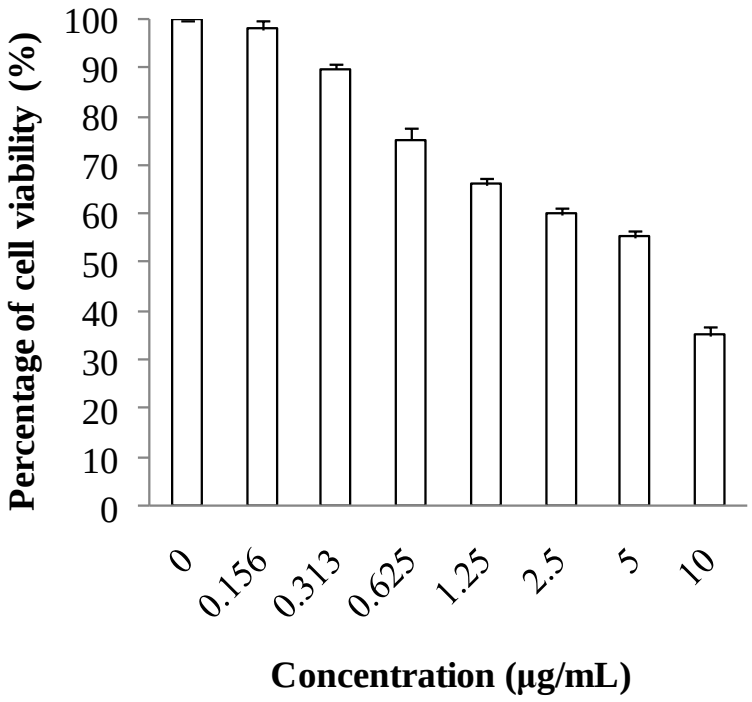


Figure 7.80 : Cytotoxicity effects of cisplatin, in combination with *L. flavescens* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Leptospermum flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.220)

Equal Variance Test: Passed (P = 0.969)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	35.319	1.553	0.897
5	3	0	55.336	1.588	0.917
2.5	3	0	60.191	1.219	0.704
1.25	3	0	66.320	1.176	0.679
.626	3	0	75.409	2.284	1.319
.313	3	0	89.869	1.280	0.739
.156	3	0	98.212	1.590	0.918
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	10782.455	1540.351	717.027	<0.001
Residual	16	34.372	2.148		
Total	23	10816.827			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	64.681	54.048	<0.001	Yes
0 vs. 5	44.664	37.322	<0.001	Yes
0 vs. 2.5	39.809	33.265	<0.001	Yes
0 vs. 1.25	33.680	28.143	<0.001	Yes
0 vs. .626	24.591	20.549	<0.001	Yes
0 vs. .313	10.131	8.466	<0.001	Yes
0 vs. .156	1.788	1.494	0.155	No

Figure 7.81 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Oxalis barrelieri* (root) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.33 ± 0.24 μg/mL. Concentration of *O. barrelieri* (root) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

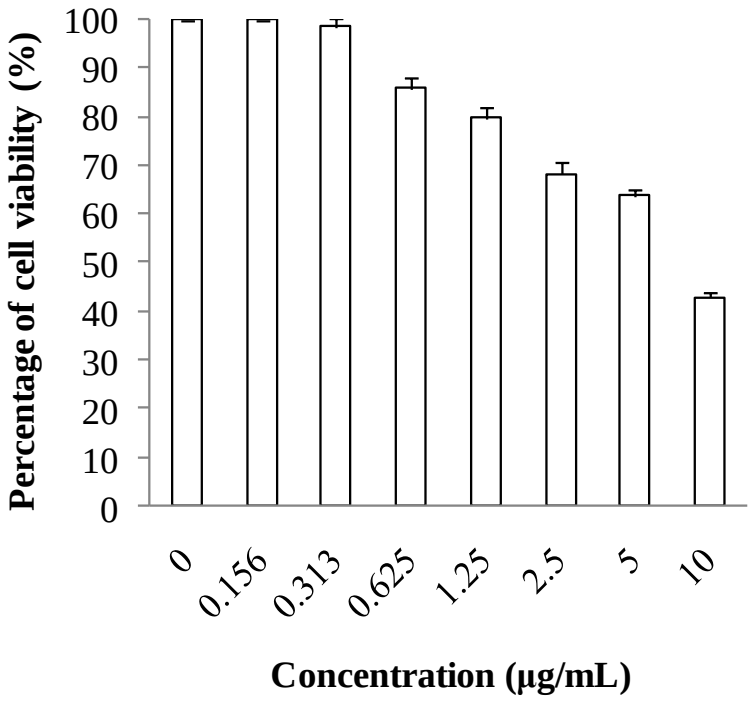


Figure 7.81 : Cytotoxicity effects of cisplatin, in combination with *O. barrelieri* (root) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Oxalis barrelieri* (root) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.086)

Equal Variance Test: Passed (P = 0.886)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	43.059	0.879	0.508
5	3	0	63.815	1.611	0.930
2.5	3	0	68.281	2.398	1.385
1.25	3	0	79.922	2.173	1.254
.626	3	0	86.006	2.307	1.332
.313	3	0	98.638	1.719	0.992
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8842.094	1263.156	456.996	<0.001
Residual	16	44.225	2.764		
Total	23	8886.319			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	56.941	41.947	<0.001	Yes
0 vs. 5	36.185	26.656	<0.001	Yes
0 vs. 2.5	31.719	23.367	<0.001	Yes
0 vs. 1.25	20.078	14.791	<0.001	Yes
0 vs. .626	13.994	10.309	<0.001	Yes
0 vs. .313	1.362	1.004	0.552	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.82 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Peltophorum pterocarpum* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 7.16 ± 0.19 μg/mL. Concentration of *P. pterocarpum* (leaf) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

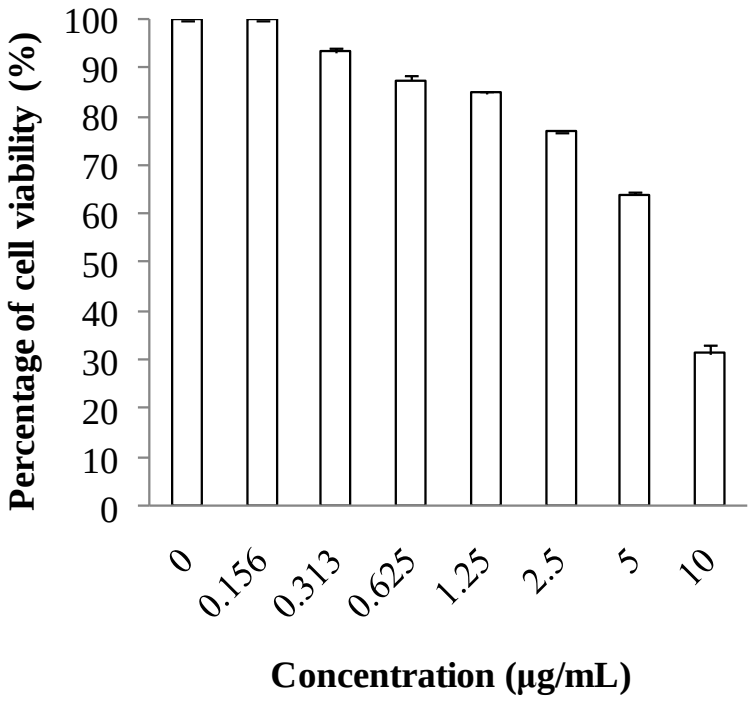


Figure 7.82 : Cytotoxicity effects of cisplatin, in combination with *P. pterocarpum* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Peltophorum pterocarpum* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	31.477	1.817	1.049
5	3	0	64.034	0.713	0.411
2.5	3	0	77.024	0.0495	0.0286
1.25	3	0	85.032	0.493	0.285
.626	3	0	87.679	0.860	0.496
.313	3	0	93.471	0.750	0.433
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	11051.394	1578.771	2358.056	<0.001
Residual	16	10.712	0.670		
Total	23	11062.106			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	68.523	102.565	<0.001	Yes
0 vs. 5	35.966	53.834	<0.001	Yes
0 vs. 2.5	22.976	34.390	<0.001	Yes
0 vs. 1.25	14.968	22.404	<0.001	Yes
0 vs. .626	12.321	18.443	<0.001	Yes
0 vs. .313	6.529	9.773	<0.001	Yes
0 vs. .156	0.000	0.000	1.000	No

Figure 7.83 shows *in vitro* cytotoxicity effects of cisplatin, in combination with *Tetracera indica* (fruit) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.47 ± 0.26 μg/mL. Concentration of *T. indica* (fruit) ethanolic extract was fixed at 50 μg/mL while concentration of cisplatin varied (0-10 μg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

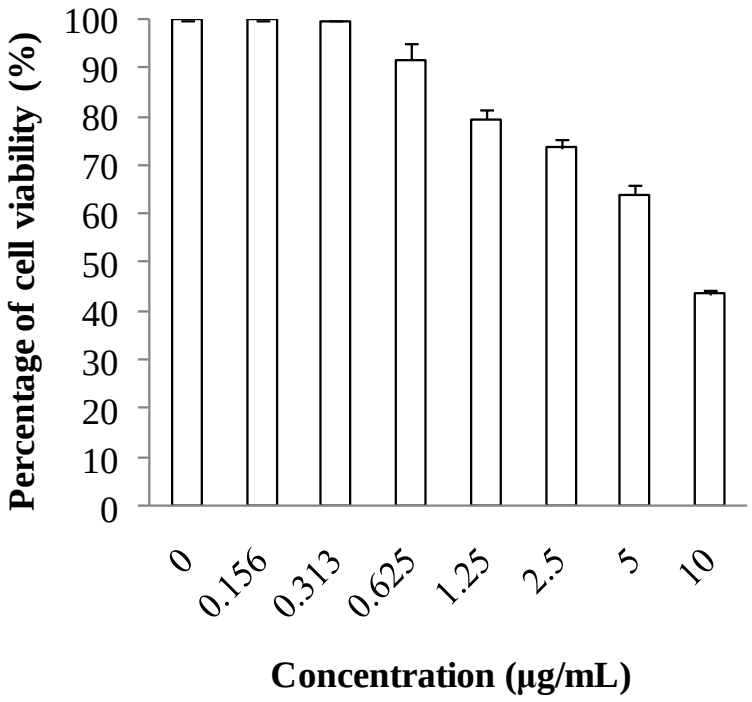


Figure 7.83 : Cytotoxicity effects of cisplatin, in combination with *T. indica* (fruit) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: *Tetracera indica* (fruit) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.073)

Equal Variance Test: Passed (P = 0.600)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	43.732	1.020	0.589
5	3	0	64.230	1.872	1.081
2.5	3	0	73.710	1.584	0.914
1.25	3	0	79.543	2.294	1.324
.626	3	0	91.812	3.479	2.009
.313	3	0	99.915	0.148	0.0853
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8756.973	1250.996	409.454	<0.001
Residual	16	48.884	3.055		
Total	23	8805.857			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	56.268	39.426	<0.001	Yes
0 vs. 5	35.770	25.064	<0.001	Yes
0 vs. 2.5	26.290	18.421	<0.001	Yes
0 vs. 1.25	20.457	14.334	<0.001	Yes
0 vs. .626	8.188	5.738	<0.001	Yes
0 vs. .313	0.0853	0.0597	0.998	No
0 vs. .156	0.000	0.000	1.000	No

Figure 7.84 shows *in vitro* cytotoxicity effects of cisplatin, in combination with 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract, on HT-29 cells. The IC_{50} value of cisplatin generated from the dose-response graph is 8.38 ± 0.36 µg/mL. Concentration of 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract was fixed at 50 µg/mL while concentration of cisplatin varied (0-10 µg/mL). Comparison of inhibition activity among concentrations using ANOVA indicates statistically significant difference with P = <0.001.

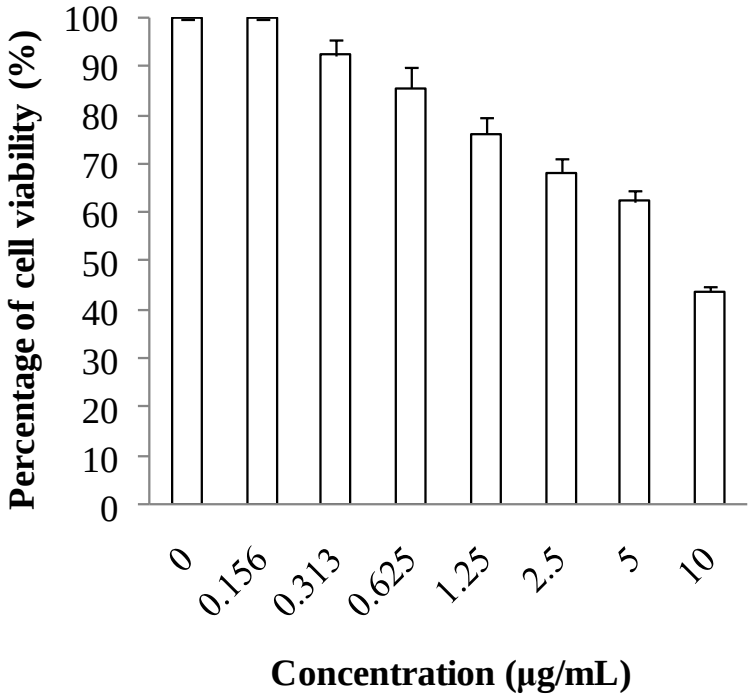


Figure 7.84 : Cytotoxicity effects of cisplatin, in combination with 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract, on HT-29 cells. Each bar represents the mean ± S.D. calculated from three independent determinations.

One Way Analysis of Variance

Data source: 50% MeOH 2% acetic acid fraction of *L. flavescens* (leaf) ethanolic extract

Normality Test (Shapiro-Wilk) Passed (P = 0.365)

Equal Variance Test: Passed (P = 0.740)

Group Name	N	Missing	Mean	Std Dev	SEM
10	3	0	44.046	0.966	0.558
5	3	0	62.509	2.219	1.281
2.5	3	0	68.472	2.683	1.549
1.25	3	0	76.378	3.514	2.029
.626	3	0	85.797	4.211	2.431
.313	3	0	92.504	3.122	1.802
.156	3	0	100.000	0.000	0.000
0	3	0	100.000	0.000	0.000

Source of Variation	DF	SS	MS	F	P
Between Groups	7	8163.980	1166.283	176.455	<0.001
Residual	16	105.752	6.610		
Total	23	8269.732			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
0 vs. 10	55.954	26.656	<0.001	Yes
0 vs. 5	37.491	17.860	<0.001	Yes
0 vs. 2.5	31.528	15.019	<0.001	Yes
0 vs. 1.25	23.622	11.253	<0.001	Yes
0 vs. .626	14.203	6.766	<0.001	Yes
0 vs. .313	7.496	3.571	0.005	Yes
0 vs. .156	0.000	0.000	1.000	No

One Way Analysis of Variance

Data source: Overall IC50 for cisplatin combination

Normality Test (Shapiro-Wilk) Passed (P = 0.390)

Equal Variance Test: Passed (P = 0.568)

Group Name	N	Missing	Mean	Std Dev	SEM	
Cisplatin alone	3	0	9.487	0.130	0.0751	
CisP + Ethacrynic acid	3	0	8.783	0.148	0.0857	
CisP + AO (branch)	3	0	8.663	0.126	0.0726	
CisP + AP (leaf)	3	0	4.697	0.180	0.104	
CisP + CZ (branch)	3	0	9.233	0.0702	0.0406	
CisP + ERi (fruit&flower)	3	0	8.547	0.0603	0.0348	
CisP + GA (branch)	3	0	8.300	0.122	0.0702	
CisP + GM (branch)	3	0	9.240	0.555	0.320	
CisP + GM (leaf)	3	0	6.737	0.376	0.217	
CisP + HT (leaf)	3	0	9.237	0.297	0.171	
CisP + LI (branch)	3	0	5.457	0.298	0.172	
CisP + LF (leaf)	3	0	6.333	0.395	0.228	
CisP + OB (root)	3	0	8.327	0.241	0.139	
CisP + PP (leaf)	3	0	7.160	0.185	0.107	
CisP + TI (fruit)	3	0	8.473	0.260	0.150	
CisP + 50% MeOH 2% acetic acid	3	0	8.380	0.361	0.208	

Source of Variation	DF	SS	MS	F	P
Between Groups	15	93.359	6.224	84.193	<0.001
Residual	32	2.366	0.0739		
Total	47	95.725			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	Diff of Means	t	P	P<0.050
Cisplatin al vs. CisP + AP (l	4.790	21.577	<0.001	Yes
Cisplatin al vs. CisP + LI (b	4.030	18.153	<0.001	Yes
Cisplatin al vs. CisP + LF (l	3.153	14.204	<0.001	Yes
Cisplatin al vs. CisP + GM (l	2.750	12.387	<0.001	Yes
Cisplatin al vs. CisP + PP (l	2.327	10.481	<0.001	Yes
Cisplatin al vs. CisP + GA (b	1.187	5.345	<0.001	Yes
Cisplatin al vs. CisP + OB (r	1.160	5.225	<0.001	Yes
Cisplatin al vs. CisP + 50% M	1.107	4.985	<0.001	Yes
Cisplatin al vs. CisP + TI (f	1.013	4.565	<0.001	Yes

Cisplatin al vs. CisP + ERi (	0.940	4.234	0.001	Yes
Cisplatin al vs. CisP + AO (b	0.823	3.709	0.004	Yes
Cisplatin al vs. CisP + Ethac	0.703	3.168	0.013	Yes
Cisplatin al vs. CisP + CZ (b	0.253	1.141	0.599	No
Cisplatin al vs. CisP + HT (l	0.250	1.126	0.465	No
Cisplatin al vs. CisP + GM (b	0.247	1.111	0.275	No

Multiple Comparisons versus Control Group (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

Comparison	**Diff of Means**	**t**	**P**	**P<0.050**
CisP + Ethac vs. CisP + AP (l	4.087	17.953	<0.001	Yes
CisP + Ethac vs. CisP + LI (b	3.327	14.614	<0.001	Yes
CisP + Ethac vs. CisP + LF (l	2.450	10.763	<0.001	Yes
CisP + Ethac vs. CisP + GM (l	2.047	8.991	<0.001	Yes
CisP + Ethac vs. CisP + PP (l	1.623	7.131	<0.001	Yes
CisP + Ethac vs. CisP + GA (b	0.483	2.123	0.321	No
CisP + Ethac vs. CisP + OB (r	0.457	2.006	0.358	No
CisP + Ethac vs. CisP + GM (b	0.457	2.006	0.322	No
CisP + Ethac vs. CisP + HT (l	0.453	1.992	0.291	No
CisP + Ethac vs. CisP + CZ (b	0.450	1.977	0.256	No
CisP + Ethac vs. CisP + 50% M	0.403	1.772	0.304	No
CisP + Ethac vs. CisP + TI (f	0.310	1.362	0.455	No
CisP + Ethac vs. CisP + ERi (	0.237	1.040	0.519	No
CisP + Ethac vs. CisP + AO (b	0.120	0.527	0.602	No

APPENDIX V: STANDARD CURVE

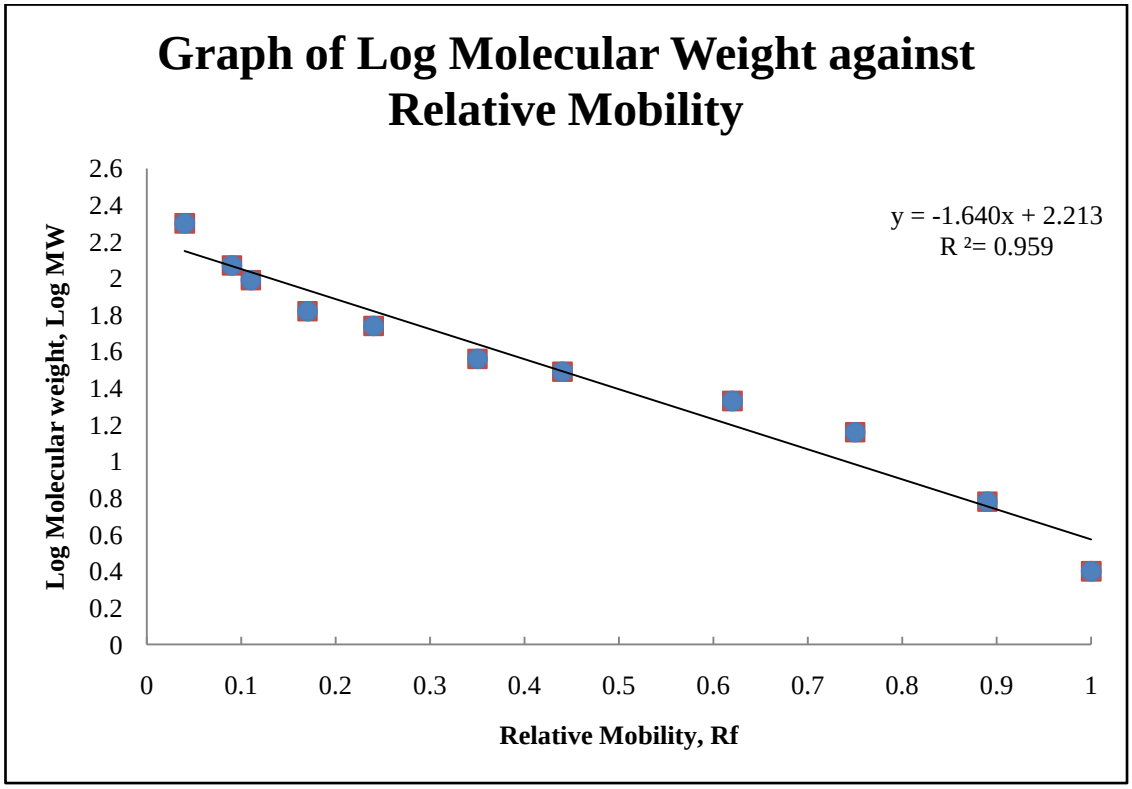


Figure 7.85 : Standard curve for SDS-PAGE protein molecular weight determination.